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Hypoglycaemia-induced changes in the patterns of breathing in rodents

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Abstract

Hypoglycaemia induces hyperpnoea and changes in breathing pattern either directly or via a metabolic intermediate. We hypothesised hypoglycaemia would augment response to hypercapnia and hypoxia equally in mice and rats but induce active expiration in hypercapnia only. Using whole-body plethysmography, the response to hypercapnia (8% CO_2), hypoxia (10% O_2) and hyperoxia (100% O_2) were recorded under normoglycaemic, fasted and insulin-induced hypoglycaemic conditions in male Wistar rats and C57BL/6 mice.

Hypoglycaemia increased baseline ventilation and CO_2 sensitivity but not O_2 sensitivity in conscious rats but had no effect in mice. Responses in anaesthetised rats were blunted and baseline frequency was reduced from 100BPM±20 to 70BPM±20. In conscious rats, the Ti:Te ratio was increased from 0.6±0.05 to 0.9±0.1 in hypercapnia indicating a active expiration that was not observed in response to hypoxia with only a increase from 0.6±0.05 to 0.7±0.1 at the same tidal volume.

In conclusion hypoglycaemia induced changes in respiration are observed in rats but not in mice. This may be attributed to differences in counter-regulatory response between species. Hypercapnia and hypoxia do not induce the same changes in ventilatory patterns.

Acknowledgements

I would like to thank D Andrew Coney and Dr Clare Ray for there help and guidance throughout this project and most of all for their patience as well which was undeniably needed at times. I would also like to thank all the members of the laboratory for making my time on this project a thoroughly enjoyable experience.

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Abbreviations

BG- Blood glucose

CB- Carotid body

CBR- Carotid body resected

CSF-Cerebral spinal fluid

CSN- Carotid sinus nerve

EEP- End expiratory pause

R_f - Frequency

V_E - Minute ventilation

NADPH- Nicotinamide adenine dinucleotide phosphate

NTS- Nucleus tractus solitaries

PEF- Peak expiratory flow

PIF- Peak inspiratory flow

T_e-Expiratory time

T_i- Inspiratory Time

V_T- Tidal Volume

VRG-Ventral respiratory Group

1.Introduction

1.1 Control of ventilation

The aim of this study is to use whole body plethysmography to examine how the pattern of breathing in response to hypoxia and hypercapnia is changed by hypoglycaemia in both rats and mice. This as is discussed in the introduction to follow will help to deduce the mechanism by which the hyperpnoea during hypoglycaemia is produced. We also look to show that plethysmography is an effective replacement for anaesthetised animal studies thus removing the variability in response that anaesthesia can have on the response to hypercapnia and hypoxia.

Minute ventilation is defined as the volume of air expired per minute (V_E), which can be altered through changes in frequency (R_f = Respiratory frequency) or the volume (V_T = Tidal volume) of breaths. Inspiration begins with the diaphragm contracting and moving down increasing the volume of the thoracic cavity. This increased volume creates a negative pressure gradient, which causes an influx of air into the lungs increasing their volume, until the sum of the elastic recoil of the alveoli, and chest wall is equal to the outward force provided by the inspiratory muscles. Expiration is normally a passive process that occurs with a relaxation of the inspiratory muscles, which causes the elastic recoil of both lungs and chest wall to exceed the outward pressure, increasing pressure in the lungs above that of the atmosphere causing air to flow out the lungs until functional residual capacity is reached (Hlastala and Berger 2001). At the end of expiration there is a period during which respiratory movement spontaneously ceases, this is known as the end expiratory pause (EEP). The length of this pause is variable depending on the respiratory rate. During hyperventilation

EEP can be decreased to aid in the increased respiratory frequency, whereas in a relaxed state it can be increase prolonging the respiratory cycle.

To increase the frequency of breathing in response to stimuli such as hypoxia or hypercapnia the time it takes to inspire (T_i), expire (T_e) or the end expiratory pause must be reduced. T_i can be decreased, with faster or deeper contractions of the diaphragm and the recruitment of the external and parasternal intercostal muscles moving the chest up and out further increasing the negative pressure gradient (Hlastala and Berger 2001). To decrease T_e the passive expiratory process must become an active one through the recruitment of expiratory muscles such as the internal intercostal muscles that act to pull the chest down, triangularis sterni and the expiratory abdominal muscles (external oblique's, rectus abdominis, internal obliques and tranversus abdominis). They act to pull the chest wall down and inward which forces air out at a quicker rate reaching residual capacity quicker (Hlastala and Berger 2001). T_i and T_e can be varied independently of each other, allowing the breathing pattern to change in response to different stimuli. Hypoxia and Hypercapnia both have different effects on both T_i and T_e (Gautier 1976b, Road, Newman, and Grassino 1986) with the change in T_e being particularly important as there is a greater reduction in T_e in hypercapnia showing a active expiration with recruitment of abdominal muscles (Jenkin and Milsom 2015). Gautier (1976) finding that anaesthesia also alters the changes in breathing patterns found in response to these stimuli.

Changes in V_E are controlled in the medulla and pons found in the lower part of the brain stem. There are two dense columns of neurons in the medulla whose activity is directly related to various phases of the respiratory cycle (Hlastala and Berger 2001). These are termed, the dorsal respiratory group (DRG), which control inspiration, and the ventral respiratory group (VRG), which control both inspiration and expiration. The DRG has projections to the lower respiratory motor neurons of the contralateral phrenic nerves controlling diaphragm contraction. Both work to control respiratory depth and frequencies with the magnitude of both being determined by stimulus from central and peripheral chemoreceptors and lung stretch receptors (Figure 1).

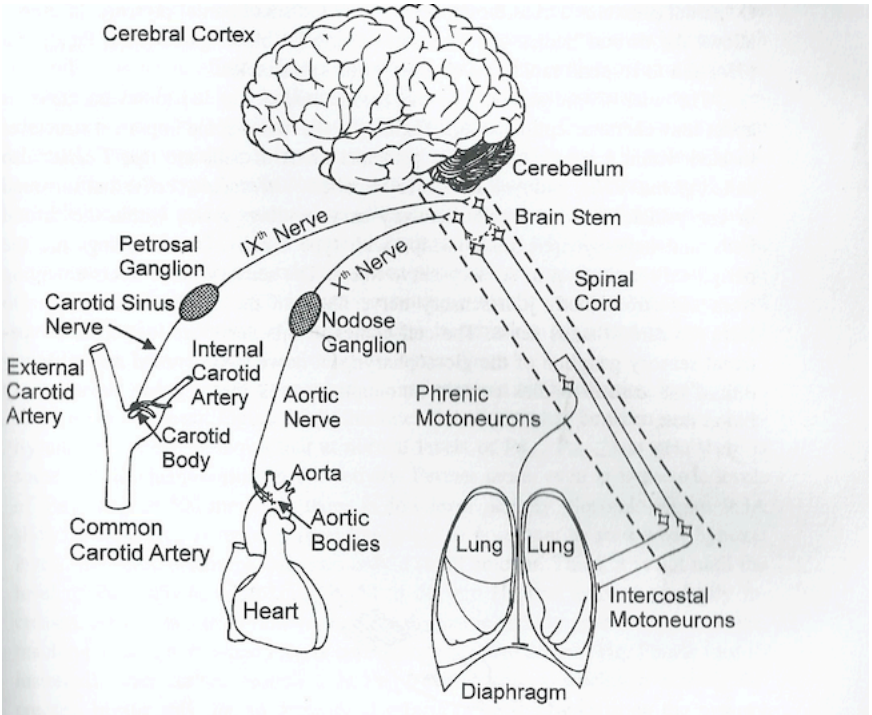

Figure 1: ***Schematic drawing of the neural pathway from peripheral chemoreceptors (carotid and aortic bodies) and lung stretch receptors to the central nervous system*** *(Hlastala and Berger 2001)*

The central chemoreceptors are located in the medulla and respond to changes in pH in the cerebral spinal fluid (CSF). Changes in pH are directly related to alterations

in the P_aCO_2 with an increase in P_aCO_2 causing an increase in the PCO_2 of the CSF, which pushes the hydration equilibrium to the right:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

The increase in H^+ is particularly sensitive in the CSF as it lacks the proteins to properly buffer this increase. This then causes hyperventilation that increases the removal of CO_2 from the blood correcting the pH balance.

1.2 The carotid body

Classically the carotid body (CB) is seen to be sensitive to hypoxemia (decreased P_aO_2), acidosis (decreased pH) and hypercapnia (increased $P_aCO_{2)}$. Hypoxemia is the main stimulus with the CB being the only chemoreceptor that elicits a ventilatory response to hypoxia, with a significant increase in discharge when P_aO_2 falls below 60mmHg. Due to the polymodal nature of the carotid body all three stimuli cause an increased V_E. However there is now evidence that points to the CB being sensitive to various blood-borne, physicochemical stimuli including catecholamines and hypoglycaemia, which will be discussed in, further detailed later.

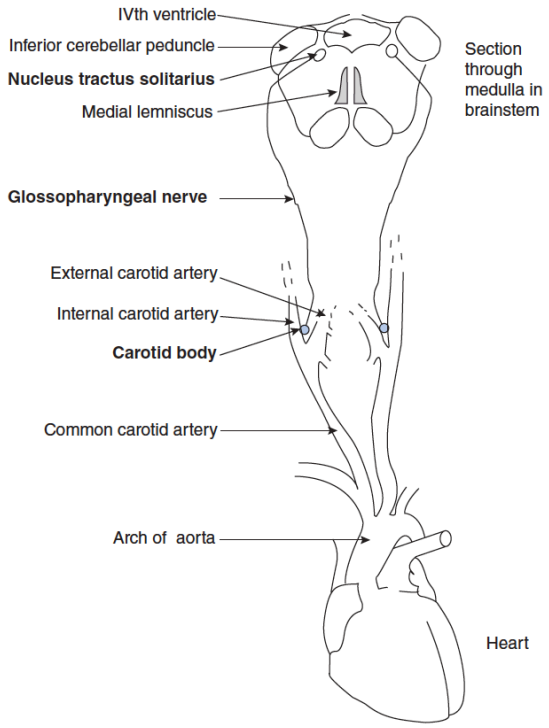

Figure 2: ***The anatomical location of the carotid body****. The carotid bodies are located, bilaterally, at the bifurcation of the common carotid arteries into the internal and externals carotid arteries. The afferent supply of the carotid body is via the glossopharyngeal nerve, which enables chemoreceptor sensory information to be transmitted to the NTS in the medulla of the brainstem, from where the information can be integrated into appropriate reflex responses mediated through respiratory motor neurons and the autonomic nervous system (Kumar, 2007).*

The carotid bodies are located bilaterally at the bifurcation of the common carotid artery into the internal and external carotid arteries. This allows them to be a highly vascularized organ with a blood flow per unit mass that is 10 times greater than that seen in the brain (Kumar and Prabhakar 2012). This high blood flow allows the maintenance of a near arterial PO_2 and facilitates its role in oxygen sensing. Upon stimulation there is a primary and in which, efferent impulses are sent through the carotid sinus nerve (CSN), a branch of the glossopharyngeal nerve, that projects to the medulla and thus connects O_2 sensing to cardiorespiratory responses including,

hyperventilation, bradycardia and peripheral vasoconstriction. A secondary response has the effect of masking the cardiovascular components as hyperventilation stimulates the pulmonary stretch receptors causing a tachycardia and vasodilation.

The carotid body is made up of two main cells types, type I (glomus) and type II (sustentacular) cells. Type I cells are the most numerous cell type with groups of 3 to 5 surrounded by cytoplasmic processes of a single type II cell forming clusters know as glomeruli (Kumar and Prabhakar 2012). Each glomeruli synapses with a branch of the CSN (Figure 3).

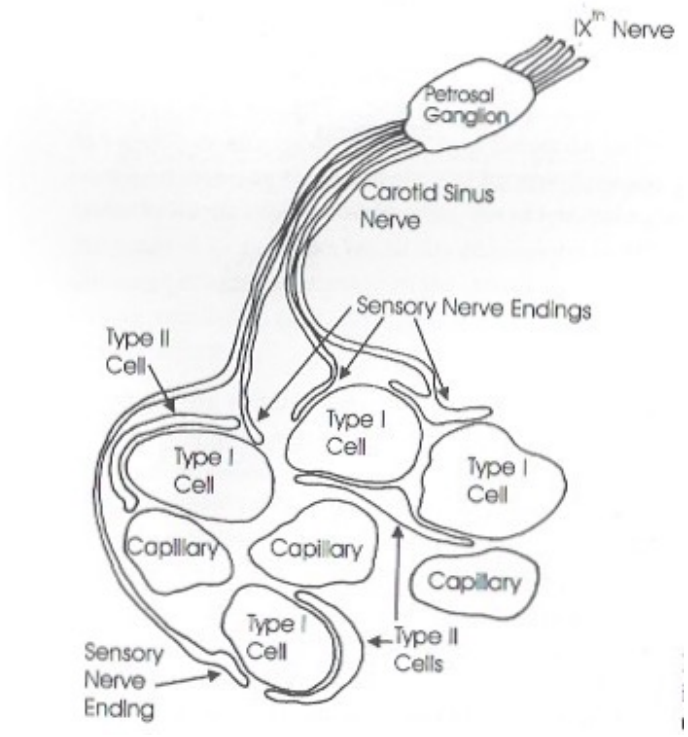

Figure 3: ***Schematic of the cellular anatomy of the carotid body****. Showing the organisation of type I and II cells into defined glomeruli, which each have a ready blood supply and nerve connections (Hlastala and Berger 2001).*

Type I cells are believed to be the chemosensory units due to synaptic contact with CSN endings and their ability to provide a response to hypoxia when isolated *in vitro* (Kumar and Prabhakar 2012), whereas type II cells provide a more supportive glial-like role. Despite the consensus that type I cells generate the chemosensory impulse, with closing of K^+ channels leading to depolarization and neurotransmitter release, the mechanism by which low O_2 leads to regulation of K^+ is still up for debate. There have been 2 hypotheses put forward; firstly that a heme/redox sensitive enzyme is the oxygen sensor and an associated biochemical event triggers the transduction. NADPH oxidase is one candidate as it is histochemically localized to the CB and has a role in the production of H_2O_2, which in turn could regulate K^+ channels (López-Barneo 2003). Hypoxia would decrease H_2O_2 production and thus lowers permeability to K^+ causing depolarisation and signal transduction. However genetic variants with a deficiency of gp91 phox subunit of NADPH still maintain the chemosensory response to hypoxia (Prabhakar 2000). Mitochondrial cytochromes have also been put forward as a possibility due to the apparent type I cell mitochondrial depolarization during hypoxia signalling and that cyanide, an oxidative phosphorylation inhibitor, mimics the effect of hypoxia. There are some issues, as it is uncertain how mitochondrial depolarisation links with increased afferent nerve activity (Prabhakar 2000). The second hypothesis suggests that K^+ channel proteins are the primary sensors and that inhibition of this channel by hypoxia is the pivotal event for depolarisation. The identification of the type of channels involved is yet to be elucidated (Prabhakar 2000). How the sensing of adrenaline and hypoglycaemia causes changes in this process is yet to be elucidated.

The CB response to P_aCO_2 provides only a limited amount of stimulus for the V_E response to hypercapnia with most drive coming from the central chemoreceptors. However, due to the high blood flow the CB response is a lot quicker than the central receptors therefore may be used for sudden increases in P_aCO_2. Similarly to a hypoxic stimulus hypercapnia leads to hyperventilation that increases the removal of CO_2 across the alveoli membrane.

1.3 Hypoxia

The PO_2 of atmospheric air is 160mmHg, which once inhaled reduces to 150mmHg due to the humidity in the airway. The partial pressure of O_2 drops to 100mmHg by the time it reaches the alveoli (P_AO_2), where it diffuses into the arterial blood (P_aO_2), bound by haemoglobin and to a limited extent dissolved in solution. Once this reaches cells and diffuses to the mitochondria the PO_2 is only around 25-30mmHg. Clearly any change in V_E will affect the alveolar and hence cellular levels of O_2 and CO_2, for example hypoventilation causes PO_2 to fall and PCO_2 to rise as metabolic use of O_2 and production of CO_2 will continue.

There is a normal basal discharge from the CB of 0.2 to 2.0Hz during normoxia with no absolute threshold for stimulus being obvious. The basal discharge is still maintained at non-physiological P_aO_2 levels of 600mmHg (Kumar and Prabhakar 2012). This increases substantially when P_aO_2 drops beneath 100mmHg and reaches a peak of around 10 to 20 times the normal baseline once it drops to 30mmHg (Kumar and Prabhakar 2012). This change in discharge frequency from hyperoxia to hypoxia can be seen in figure 4. Below 20mmHg there is a small drop in discharge giving a more sigmoidal nature to the response curve. This drop is due to

the need for oxygen to provide the energy needed for the maintenance of action potential, thus the failure of aerobic respiration has a negative effect on the cells ability to maintain signalling.

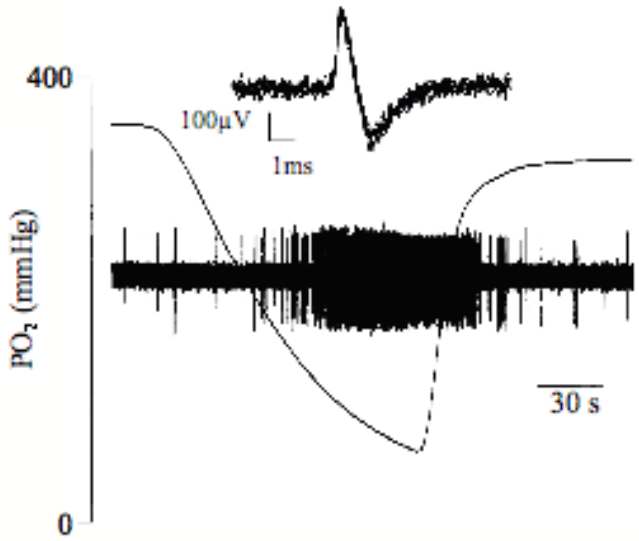

Figure 4: ***A raw trace showing in vitro chemodischarge of the CSN****. Discharge increased upon decreasing PO_2 from hyperoxia to hypoxia, which was reversed on return to hyperoxia (Kumar and Prabhakar 2012).*

The stimulation causes a Ca^{2+} dependent neurotransmitter release and CSN stimulation, which increase exponentially with the strength of the stimuli. This causes a cardiorespiratory response including hyperventilation, increasing V_E above metabolic demand, which decreases P_ACO_2, and increases P_AO_2 and P_aO_2. The cardiovascular component of the response is used to increase blood flow to hypoxic tissues, with tachycardia and peripheral vasoconstriction caused by an increased in sympathetic outflow from the medulla.

Some species of small mammals respond to hypoxia by inducing a hypometabolic state, which occurs with the depression of shivering and non-shivering thermogenic response (Mortola et al. 1994). This hypometabolic state is most pronounced in smaller mammals and new-borns that have a higher thermogenic requirement. Mortola at. al. (1994) found that the hypometabolic response to hypoxia was inversely correlated with weight in rats. This depression of the thermogenic component of metabolic was also found in a comparative study of different species including rats and mice with a general conclusion being drawn that hypoxia decreases metabolic rate with larger effects in smaller animals, because of their higher thermogenic requirements (Frappell et al. 1992). This decrease in metabolic rate will cause a reduction in the O_2 required to maintain cellular metabolism making the animal less sensitive to hypoxic stimuli.

1.4 Hypercapnia

Although not seen as its primary role the carotid body is thought to account for 30 to 50% of the respiratory drive found during hypercapnia (Kumar and Prabhakar 2012) although this could be due to chemoreceptor gain of the CNS with a CNS–CB interaction resulting in hyperadditive ventilatory responses to central hypercapnia (Blain 2010). Carotid body resection also leads to a slower response time to hypercapnia showing that the carotid body response to P_aCO_2 may be a rapid response mechanism which could have a critical role in the feed forward response to exercise (Kumar and Prabhakar 2012). Unlike hypoxia, hypercapnia signalling has a threshold with no signalling occurring below a P_aCO_2 of 18mmHg. The chemoafferent response to hypercapnia increases linearly with P_aCO_2 from 18-25mmHg until it

reaches 65mmHg where it plateaus. The response is characterised by an initial fast overshoot within one second which then falls to a lower level where it plateaus after 5-10 s (Kumar and Bin-Jaliah 2007). The signal threshold is dependent on the P_aO_2 as the threshold decreases linearly with P_aO_2 to an extent where no matter how low P_aO_2 it is possible to abolish the afferent activity with an appropriate reduction in P_aCO_2.

Chemoreceptor sensing of P_aCO_2 is believed to take place subsequent to concurrent acidification following a carbonic anhydrase dependent hydration of CO_2, which causes a drop in the intracellular pH of type I cells leading to depolarisation (Kumar and Bin-Jaliah 2007).

Hypoxia and hypercapnic signalling interact positively to produce a discharge frequency which is greater than the sum of the two responses, with Kumar and Prabhakar (2012) postulating that increasing PCO_2 may augment the sensitivity of the carotid body to hypoxia and equally hypoxia increases CO_2 sensitivity. It is possible to investigate the response to CO_2 by inhalation of a gas mixture containing increased CO_2 concentrations, which in humans it has been shown that V_E increases 2-3Lmin^{-1} for each 1mmHg increase in PCO_2.

1.5 Glucoregulation

Glucose is an important biological fuel to all cells especially neurons which use glucose as there main fuel source. Glucose is broken down through oxidative phosphorylation to form ATP, which is then used to drive processes such as enzyme driven reactions and active transport. This is particularly true for the brain, which is

unable to use any other substrate for energy. Due to this it is critical that blood glucose is maintained at normal levels (~4-7mmolL^{-1}) to allow a ready supply for the brain and other organs, this is tightly controlled through specific glucose sensitive cells, which produce a neuroendocrine response to correct any deviation from normal. These glucose-sensing cells are located in peripheral locations such as β-cells of the pancreas, hepatic portal vein, liver cells, and possibly the carotid body. Central glucose sensing is also mediated in the brain stem and hypothalamus.

A drop in blood glucose below 4 mmolL^{-1} is termed hypoglycaemia. Hypoglycaemic activation of peripheral and central glucosensors sends signals to the hypothalamus activating afferent autonomic nerves that stimulate the release of adrenaline and noradrenaline from the adrenal medulla, glucagon from the alpha cells of the pancreas and blocks the insulin secretion from ß-cells. Adrenaline and noradrenaline act on the liver via $ß_2$ adrenergic receptors to increase hepatic glycogenolysis and gluconeogenesis (Sprague and Arbeláez 2011). Glucagon binds to the glucagon receptor on peripheral and liver cells to increase the breakdown of glycogen and decrease glycogen production. Glucagon and adrenaline work in concert, with studies showing an additive effect of adrenaline and glucagon on glucose production (Sprague and Arbeláez 2011).

This process is even further controlled by the hormones cortisol and growth hormone, which act over longer time periods to that of glucagon and adrenaline. Cortisol has an additive effect on glucagon's action by exerting an anti-insulin effect reducing the uptake of glucose into peripheral tissue and increasing the break down

of proteins into amino acids, which can then be converted to glucose through gluconeogenesis. Growth hormone causes an increase in glycogen break down and lipolysis, increasing the free fatty acids available for cells to use as fuel.

An increase in blood glucose is known as hyperglycaemia and stimulates the release of insulin from the pancreatic ß-cells. Insulin has the opposite effect to that of glucagon in which it localizes the glucose transport GLUT-4 to the plasma membrane of peripheral cells increasing their uptake of glucose. This combined with increase glycogen production and increasing lipogenesis leads to a removal of glucose from the blood into storage within the cells. This process is summarised in figure 5.

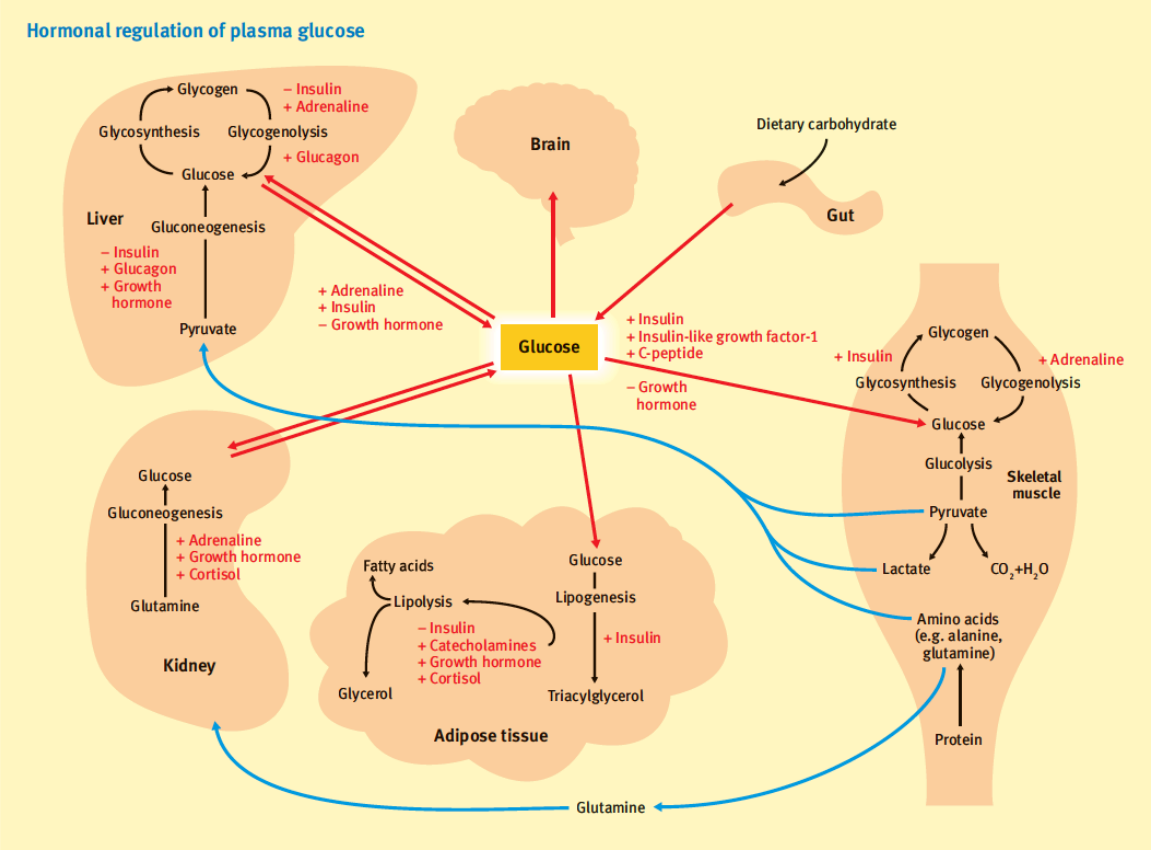

Figure 5: ***Summary of the neuroendocrine control of glucose***. With insulin shown to promote the storage of glucose during hyperglycaemia. Whereas adrenaline, cortisol

and glucagon promote the formation of glucose in response to hypoglycaemia.(Casey 2003)

1.6 The carotid body's role in glucoregulation

The carotid body has been suggested to have a role in glucoregulation as it has unique characteristics that give it the potential to be a glucose sensor. A high blood flow gives it a supply of glucose that is near that of arterial levels, this combined with its high metabolism make it sensitive to any fall in blood sugar. Extensive innervation with the circuitry to provide input into the centres involved in glucoregulation also make it a prime candidate as a glucose sensor Koyama et al.(2000). Its location on the carotid artery, which is the primary blood supply to the brain, also makes it sensitive to the level of glucose supplied to the brain. This has been confirmed by studies such as those conducted by Alvarez-Buylla and de Alvarez-Buylla (1988) who showed that direct carotid chemoreceptor stimulation by sodium cyanide increased hepatic glucose output and brain glucose retention. However there is some disagreement as to whether glucose sensing is through direct action of glucose on the carotid body or indirectly through a metabolic intermediate.

In vitro studies have shown a variation in results on the direct action of glucose on the carotid body Holmes et al. (2014) found that there was no increase in sensory output from both isolated type I cells and intact carotid bodies. They attributed this to glycogen stores in type I cells protecting against hypoglycaemia. The carotid body has also been shown to have glycogen store's that protect against hypoglycaemia in the results gained by Conde et al. (2007). They investigated the release of catecholamines using freshly isolated intact CB preparations and the electrical

activity in the CSN. There was no change in catecholamine release when 5mM or 0mM of superfusate glucose was applied, and this was confirmed with no change in the basal activity of the CSN during 0mM glucose perfusion. However they did find that after 70mins of 0mM there was an increase in catecholamine release (Figure 6).

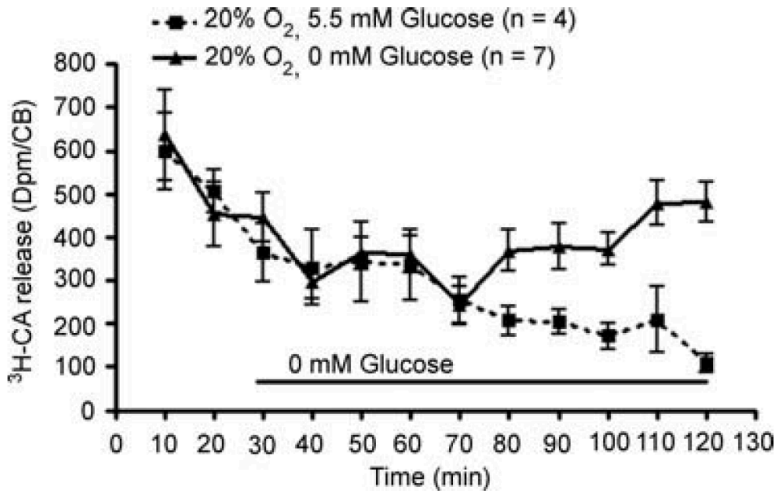

Figure 6: ***Effect of long-term application of 0 mM glucose on the basal ^{3}H-CA release from rat carotid body*** *Mean (±S.E.M.) time course of [^{3}H]CA basal release from rat CB incubated with 5.5 mM glucose (controls, continuous line; n = 4) and from rat CBs incubated with 5.5 mM glucose for the initial 30 min and with 0 mM glucose between minutes 30 and 120 (Conde, Obeso, and Gonzalez 2007)*

Carotid body resected (CBR) dogs had a flawed regulatory response to insulin-induced hypoglycaemia (Koyama et al. 2000). With a reduced endogenous glucose production (Figure 7) and a reduced plasma glucagon and cortisol response when compared to control groups. The conclusion was that this was a direct action of glucose on the carotid body however the change in counter regulatory hormones could not be ruled out as having a critical role.

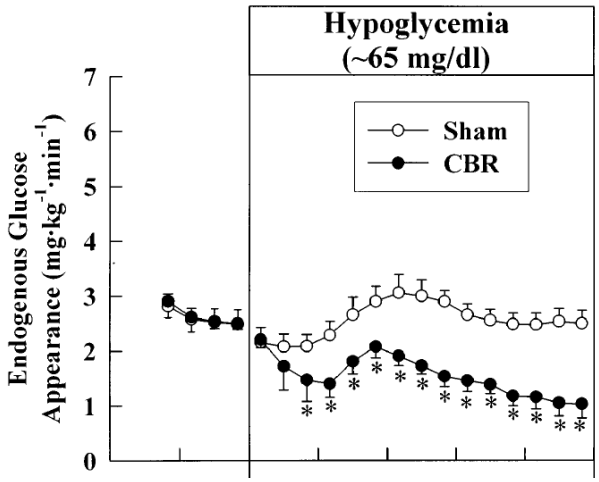

Figure 7: ***A graph showing the rate of glucose appearance during a maintained hypoglycaemic clamp in conscious dogs****. CBR (carotid body resected) animals had a lower appearance rate compared to shams, indicating a role for the CB in glucoregulation. Taken from Koyama et al (2000).*

Further research by the same lab on the glucoregulatory response during exercise in CBR dogs saw the same mismatch in endogenous glucose production and blunted glucagon and noradrenaline counteregulatory response (Figure 7 (Koyama et al. 2001).

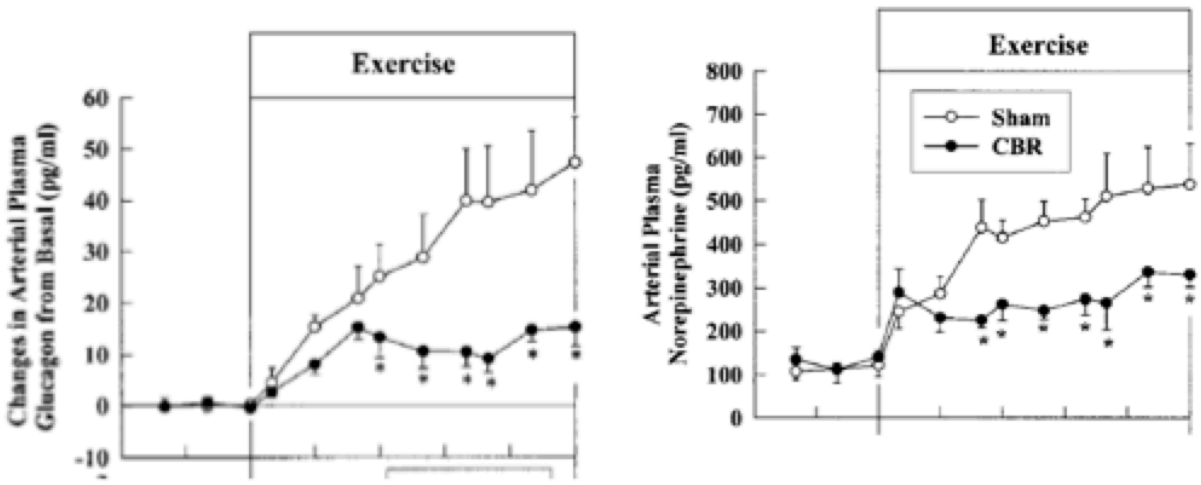

Figure 7: ***Effect of carotid body removal on arterial plasma glucagon and norepinephrine concentrations in CBR and Sham dogs****. Taken from (Koyama et al. 2001)*

The CB is a polymodal sensor with no distinguishable difference between the sensory output if it is activated by hypoxia, hypercapnia or acidosis, thus if it is indeed sensitive to hypoglycaemia then the same cardiorespiratory responses should be seen. Using this principal Bin-Jaliah et al. (2005) induced hypoglycaemia in control and CBR rats, normal controls saw an increased V_E during hypoglycaemia with a 50% increase in CO_2 chemosensitivity. However, there was no increase in V_E in CBR rats, leading to increase in P_aCO_2 causing a respiratory acidosis during hypoglycaemia. This result when combined with their later results showing there was no direct glucose stimulation of the carotid body *in vitro* (Holmes et al. 2014), suggest that the CB role in glucoregulation is to match ventilation with changes in metabolism with Bin-Jaliah et al (2005) suggesting that this may be mediated by adrenaline.

Adrenaline was identified as a possible candidate for the hormonal stimulation of carotid body during hypoglycaemia as it is released from the adrenal medulla during hypoglycaemia and is also known to increase in cellular metabolism. This increase in cellular metabolism would cause a change in blood gas levels if not matched by an increase in ventilation. The dual role for adrenaline was shown by Whelan and Young (1953) who found that infusion of adrenaline caused an increased minute ventilation and tidal volume in humans, with other experiments also showing a similar response in anaesthetised animals (Young 1957). It was proposed that this action could be through a direct stimulation of the respiratory centres or through the increase in blood flow to the carotid body that would increase their sensitivity. However the hyperpnoea in response to adrenaline could be removed through carotid body resection or application of hyperoxia (Joels and White 1968).

Exposure to hyperoxia causes an impaired carotid body response to hypercapnia and cyanide stimulation. With a reduced Ca^{2+} influx in type I cells and a decrease in the afferent nerve conduction (Kumar and Prabhakar 2012). With this in mind Wehrwein et al (2010) used hyperoxia to blunt the CB response to insulin-induced hypoglycaemia. In their studies they observed that an increased infusion rate of glucose was needed to maintain blood glucose during exposure to hyperoxia (Figure 8). The increase in glucose infusion rate shows a decrease in endogenous glucose production resulting from a reduced neuroendocrine response that was also reported.

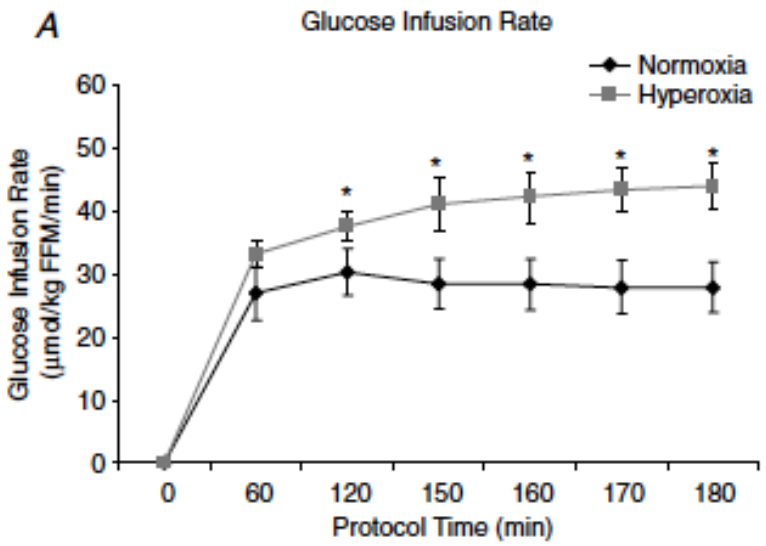

Figure 8: ***Time course for glucose infusion rate in normoxia and hyperoxia****. Values are means ± S.E.M. Significance denoted as* $*P < 0.05$ *(Wehrwein et al. 2010)*

The use of hyperoxia to silence carotid body response has some flaws as hypoglycemia increases sympathetic outflow in humans, but hyperoxia suppresses sympathetic nerve traffic. This could lead to a general non-specific sympathoinhibition that dampens the sympathetic driven response to hypoglycemia such as those that release glucagon and cortisol. However due to the reduction in

growth hormone release which is a non-sympathetic counter regulatory hormone this may not be the case (Wehrwein et al. 2010).

Like others Wehrwein et al. (2010) concluded that this was through a direct stimulation of glucose on the carotid body. However in a combined *in vitro* and *in vivo* study a lack of sensory output was found when isolated CB's were exposed to low glucose *in vitro* (Bin-Jaliah, Maskell, and Kumar 2004). But in their linked in vivo study they found that respiration was increased during insulin-induced hypoglycaemia only in sham-operated animals. There was also a marked respiratory acidosis in CBR animals, thus pointing towards a mechanism that ties respiration with an increased metabolism during insulin-induced hypoglycaemia.

1.7 Whole body plethysmography

Measuring ventilatory responses *in vivo* gives rise to certain problems as most anaesthetic agents have effects on the ventilatory system with many causing a depression of ventilation, with urethane (Maggi and Meli 1986) halothane (Davies, Edwards, and Lahiri 1982) and diazepam (Gross 1986) leading to a blunted reaction to both hypoxia and hypercapnia. With this in mind a method where subjects were conscious would be advantageous.

Whole body plethysmography is a non-invasive indirect method of measuring ventilation in conscious freely moving animals. There is no need for anesthesia or tracheal instrumentation, and it minimizes the stress effects of restraint, allowing animals to exhibit a normal breathing pattern. It works on the concept that inhaled air

is warmed and humidified when it enters the body, causing the air to expand and the pressure in the plethysmograph to rise. This change in pressure can then be compared to a reference chamber and the standard parameters of pulmonary function can be calculated. To eliminate the influence of a increase of temperature and humidity inside the chamber (Figure 9):

1. This is achieved by using a ventilation pump to draw air out of the subject chamber at a constant rate
2. Two pneumotachographs are the only routes available for entry of air into the plethysmograph.

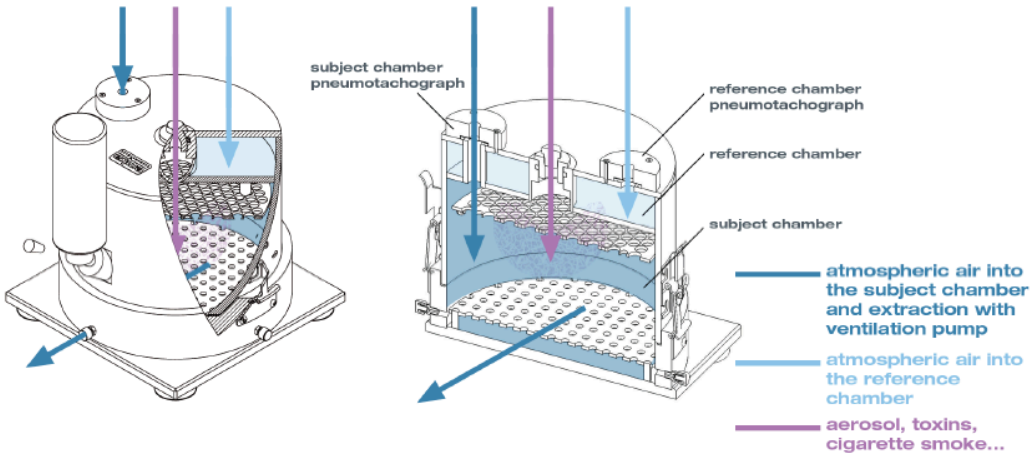

Figure 9: ***Diagram representation of the flow of air in and out of the plethysmography chamber****.*

Even though respiratory rate and tidal volume are the primary respiratory parameters, whole-body plethysmography also allows the analysis of peak flows and cycle times of inspiration (T_i) and expiration (T_e) and end expiratory pause (EEP) (Glaab et al. 2007).

1.8 Hypothesis

The CB is a peripheral chemoreceptor responding to changes in arterial gas tensions and pH. It has also been proposed to have a role in glucoregulation, although it is unknown whether the direct stimulus is low glucose or some other blood-borne mediator.

Based on the current evidence, this project aimed to further investigate how the patterns of ventilation change in hypoglycaemia. With the use of whole body plethysmography in conscious freely moving rodents we look to see how varying glycaemic levels effect the ventilation variables during exposure to hypoxia, hypercapnia and hyperoxia.

The hypotheses are as follows:

1. Hypoglycaemia will cause an increase in basal $V_{E.}$
2. The R_f response to hypercapnia will induce an active expiration, which will be shown by a decrease in T_e .
3. Hypoglycaemia will augment both hypoxic and hypercapnic responses, due to the polymodal nature of the carotid body.
4. Anaesthesia will depress baseline ventilation blunting responses to hypoxia, hypercapnia and hypoglycaemia.
5. Rats and mice will have a similar response to hypoxia, hypercapnia and hypoglycaemia.

2. Methods

2.1 Human active expiration studies

Prior to the start of the main study the change in expiratory time (T_e) during an active expiration was investigated in a human subject. This was done with the aim of being able to use this to deduce if there is an active expiration during hypercapnia and if so, does this differ between anaesthetised and conscious animals.

2.1.1 Simulated active expiration

Active expiration was carried out on myself using a respiratory flow head connected to a PowerLab and Labchart software (ADInstruments) on a Mac OS X computer. Baseline readings were taken over a 5min period. The active expiration were conducted every 20s over another 5min period directly after baseline. Inhalation before the active expiration was kept as similar to normal breathing with no change in inspiratory time (T_i).

2.2 Whole body plethysmography studies

2.2.1 Animals

Male Wistar rats and C57BL/6J male mice were sourced from [redacted] with body weights ranging from 290g to 450g for rats and 24g to 26g for mice, on the days of experimentation. The animals were housed in groups of 4, at a temperature of 21±1°C and humidity of 40-45%, under a 12-hour light/dark cycle (lights on at 7:00am). Food and water was available *ad libitum*, except before fasted and hypoglycaemic recordings when food was withdrawn from midnight before experimentation to ensure stable and consistent basal blood glucose levels and low endogenous insulin. The care of the animals and all procedures performed were

carried out in line with the Home Office (UK) guidelines, University of Birmingham guidelines on ethical use of animals and the Animals (Scientific Procedures) Act (UK) 1986.

2.2.2 Equipment and data collection

Rat and mouse whole body plethysmography chambers [redacted] were used for conscious unrestrained recordings of ventilatory variables. The chambers were contained in an ante-room away from the computer and experimenter to remove any visual or audible stimuli that may provoke movement. To enable the monitoring of the animals activity a video camera was set up above the chamber with a link back to the test computer. The chamber was calibrated daily before the experiments with an integration calibration using the sealed chamber as low value and an injection of 5ml of air for the high value. Gas extraction was set at $2mls^{-1}$ for rats and $0.5mls^{-1}$ for mice, which was controlled through a Vent 2 (Emka) air pump. Chamber gas mixtures were changed using the Iox 2.8.0.17 software and mass flow analysers (Alicat Scientific). The mixtures were pumped into the chamber using a vent 4-C1 (Emka) at a rate equal to the rate of extraction, with all gas (N_2, O_2 and CO_2) coming from gas cylinders (BOC). Data was recorded using a differential pressure transducer (Emka) at a sample rate of 200Hz. Direct measures of ventilation (V_T, T_i, T_e and Peak expiratory flow + Peak inspiratory flow) were calculated from flow trace (Figure 11) with further measures such as frequency ($R_f=60/T_I+T_e$) and minute volume ($V_E=V_T*R_f$) calculated by the Iox 2.8.0.17 software (Emka) installed on a Dell OptiPlex 990 Windows 7 computer.

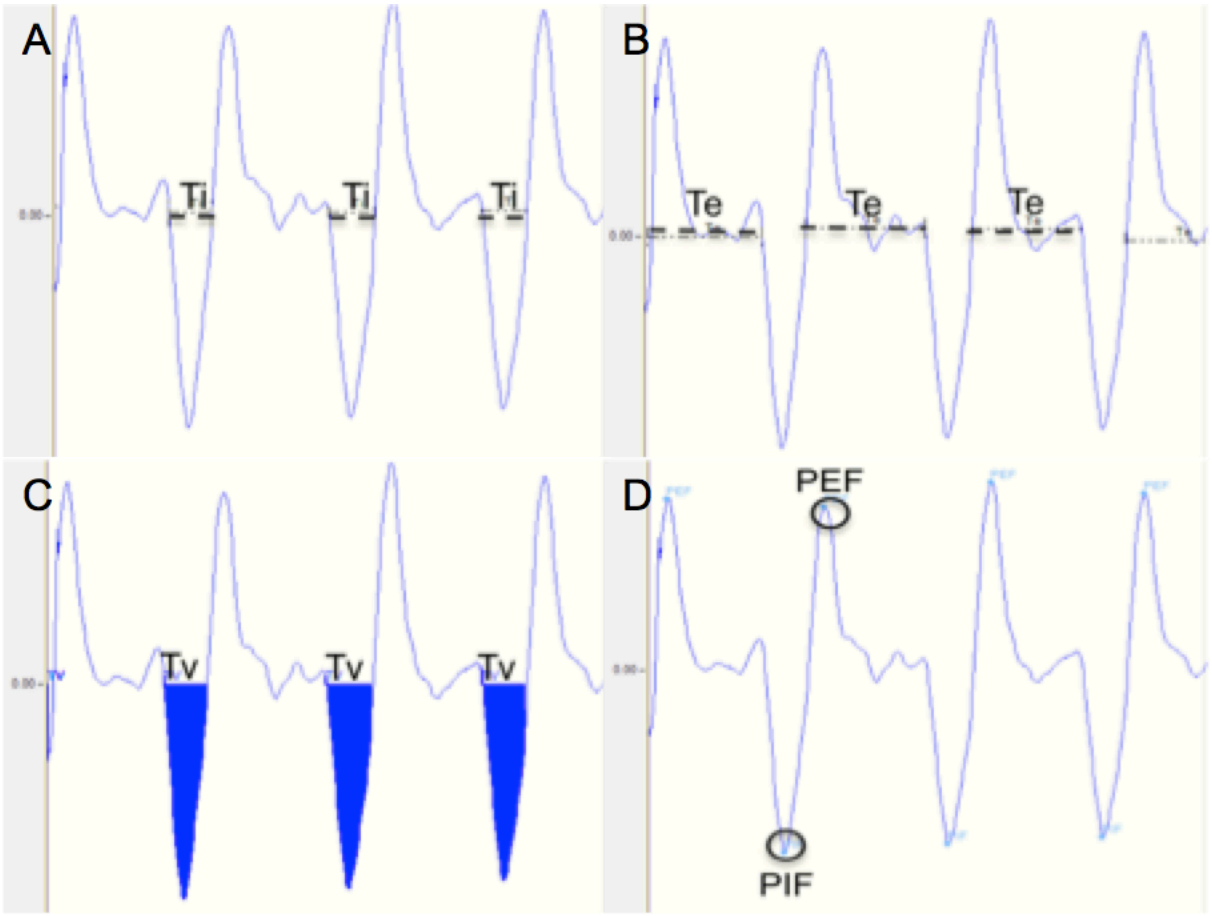

Figure 11: **Flow traces depicting how the lox software calculates respiratory variables**. A) T_i is calculated using the integral inspiratory phase (negative deflection) to return to zero. B) T_e integral of expiratory phase (positive defection) to return to zero. C) V_T is the area under the inspiratory peak. D) PEF and PIF peak positive and negative deflection.

Exclusion criteria were also set up to exclude any noise caused by movement of the animal that may be registered as a breath by the software. The exclusion limits are shown in table 1 and were recommended in the lox whole body plethysmography manual as being outside of mouse and rat physiological limits.

Parameter	Mouse		Rat	
	Min	Max	Min	Max
T_i (ms)	60	1000	100	2000
T_e (ms)	80	1000	120	2000
V_T (ml)	0.04	10	0.4	10
R_f (BPM)	10	450	10	250

Table 1: **Exclusion criteria used to remove noise from calculated parameters as recommended by the lox whole body plethysmography user manual**.

2.2.3 Normal and Fasted Protocol

A sub group of 4 rats were given 5days of acclimatisation in the plethysmography chamber to see if this had any effect on ventilation. The rest of the animals were acclimatised to the chamber for 30mins the day before the experiment started, after which animals completed the following protocol in a normoglycaemic, fasted and insulin-induced hypoglycaemic states (see below). The animals were placed in a warming chamber (Fuji electric) at 41^{o}C for 5 to 10mins after which blood glucose was measured using an Aviva Nano (Accu-Chek®) blood glucose monitor with blood being obtained through a tail prick. Baseline measurements were taken until there was 5mins of stable readings; this took between 15 and 30mins depending on the activity levels of the animals in the chamber. They were then exposed to 5mins of either hypercapnic (21% O_2, 71% N_2, 8% CO_2) or hypoxic (10% O_2, 90% N_2) air. Their ventilatory variables were then allowed to return to baseline and after a further 5mins they were exposed to hypercapnic or hypoxic air depending on the previous exposure. Finally they were exposed to hyperoxic (100% O_2) conditions for 30s. The order of the exposures was switched during each session so no rat was exposed in the same order to the previous day. Due to the length of the protocol the order at

which the rats were exposed was changed each day to allow for any role their circadian rhythm may have had on the responses.

2.2.4 Hypoglycaemia

Two out of four animals were fasted overnight (12:00pm to 9:00am) this was accomplished through moving the animals into separate cages, in pairs for mice and single cages for rats from 3:00pm the day before with only 10g of food provided for rats and 5g for mice. A blood glucose reading was taken after which an IP injection of Bovine insulin (CP Pharmaceutical Ltd.) was then given in a dose of $1.5IUKg^{-1}$ for rats and $1IUKg^{-1}$ for mice. Animals were then placed in the plethysmography chamber for 30mins while ventilation was recorded. After 30mins a second blood glucose measurement was taken and animals were returned to the plethysmography chamber and exposed to hypercapnia, hypoxia and hyperoxia following the same protocol as previously stated. Once the exposures were finished they were removed from the chamber and a third blood glucose reading was taken.

2.3 Anaesthetised Rat Studies

2.3.1 Surgical procedures

Anaesthesia was induced with 4% isoflurane in O_2 at 4 L min^{-1} (Merial Animal Health Ltd.). The right jugular vein was isolated and cannulated, allowing maintenance of anaesthesia with a 17-20 $mgkg^{-1}hour^{-1}$ infusion of Alfaxan® (Vétoquinol UK Ltd.) using a syringe driver (Perfusor® securaFT, B. Braun.), and 0.1ml boluses as necessary. The trachea was isolated and cannulated with a stainless steel T-piece cannula. The left and right femoral arteries were isolated from the femoral sheaths and cannulated.

Depth of anaesthesia was monitored through response to a strong paw pinch. Core body temperature was also monitored and maintained at 37°C throughout, via a rectal temperature probe linked to a homeothermic heat pad system (Harvard Apparatus).

2.3.2 Equipment

All cannulae were filled with heparinised saline (20IUml^{-1} Heparin, LEO® Pharma) and sourced from Portex™, Smiths Medical. Suture was from Look®, Surgical Specialities Corporation and needles (0.5x25, 0.6x30mm and 0.8x40mm, Microlance™) and syringes (1, 2, 5 and 10ml, Plastipak™).

A spirometer (MacLab, ADInstruments) was attached to the tracheal T-piece to measure airflux, allowing calculation of respiratory frequency (R_f), tidal volume (V_T) and minute ventilation ($V_E = R_f \times V_T$). A plastic tube running from cylinders of N_2, O_2 (BOC) and CO_2 (BOC), was attached to the spirometer via the t-piece, allowing control of inspired gasses by a rotameter (Cole Parmer).

A pressure transducer (Capto) was attached to the right femoral artery cannula to monitor arterial blood pressure (ABP) (via a MacLab Bridge Amp, ADInstruments), from which MABP and HR were derived. Blood samples were taken via left femoral artery cannula and used to measure blood glucose levels using Aviva Nano (Accu-Chek®) blood glucose monitor

2.3.3 Protocol

Data was recorded at sampling frequencies of 100-1000Hz, using PowerLab and Labchart software (ADInstruments) on a Mac OS X computer. A 30min period was allowed before recordings were started to allow recovery from surgery. A 10min

baseline was then recorded after which each animal was exposed to a 5min exposure to hypercapnia (21% O_2, 71% N_2, 8% CO_2) they were then allowed to return to baseline levels for 5mins after which they were exposed to hypoxic stimulus for a further 5mins (10% O_2, 90% N_2). Finally after returning to baseline they were exposed to a 30second hyperoxic stimulus (100% O_2).

An IP injection of insulin (1.5IUKg^{-1}) was then given and allowed 30mins to take effect after which a blood glucose measurement was taken and the gas exposures were repeated as before. Blood glucose was then measured at the end of the experiment, after which the animal was [redacted].

2.4 Data collection and analysis

All analysis and graphing was conducted using GraphPad Prism 6.0 for Mac. V_T, R_f, T_i, T_e and EEP data are all shown as mean ± SD. Baseline was calculated as the mean over the 5mins proceeding exposures and compared to the last minute of exposure in hypercapnia and hypoxia using a Student's paired t-test. Hyperoxic responses were calculated with a mean over the full 30 s of exposure. All data were analysed to the 95% significance level i.e. $P < 0.05$ taken as significant.

Differences between baselines and responses in normal, fasted and hypoglycaemic tests were investigated using a repeated measures one-way ANOVA with Bonferroni's post hoc test.

Relationship between blood glucose level and ventilatory variables at baseline and during responses were plotted as scatter graphs. Linear regression lines were added and analysed for their difference from zero and the goodness of fit in the data.

CO_2 and O_2 sensitivity was calculated from the change in V_E from baseline in response to hypercapnia and hypoxia. This was then divided by the level of gas exposure (10% for O_2 and 8% for CO_2) at each of the glycaemic levels. These were then compared with a one-way repeated measure ANOVA with Bonferroni's multiple comparison.

3. Results

3.1 Human Active Expiration Investigation

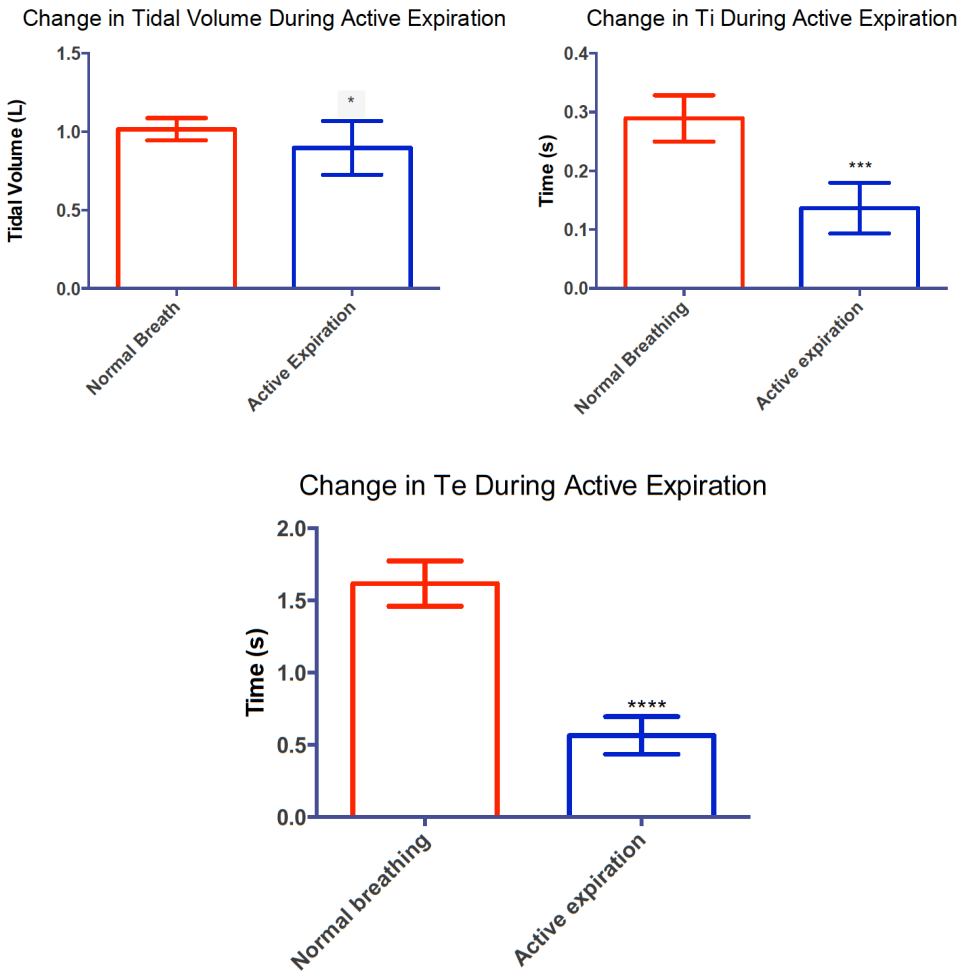

Figure 12: ***The change in V_T, T_i and T_e during a simulated active expiration****. Mean ±SD compared with a Student's t-test. ***P≤0.001, *P≤0.05.*

Despite attempts to maintain a constant breathing pattern both tidal volume (P=0.0282) and inspiration time (P< 0.0001) were significantly decreased in active expiration breaths when compared with normal breathing (Figure 12). Figure 12 shows that as expected T_e was significantly decreased during active expiration from a mean of 1.617ms ± 0.04355 to 0.5657ms ± 0.03364.

3.2 Rats Physiological characteristics

	Time From Start of Study	Weight (g)
Acclimatisation	0 days	297.6 ± 9.7
Normal	9 days	333.5 ± 12.6
Fasted	16 days	348.29 ± 18.0
Hypoglyceamic	29 days	364.71 ± 42.79
Anaesthetised	39 days	414.43 ± 16.65

Table 2: ***Body weight at each point in testing.*** *Mean ± SD. Animals grew normally during the experimental protocol.*

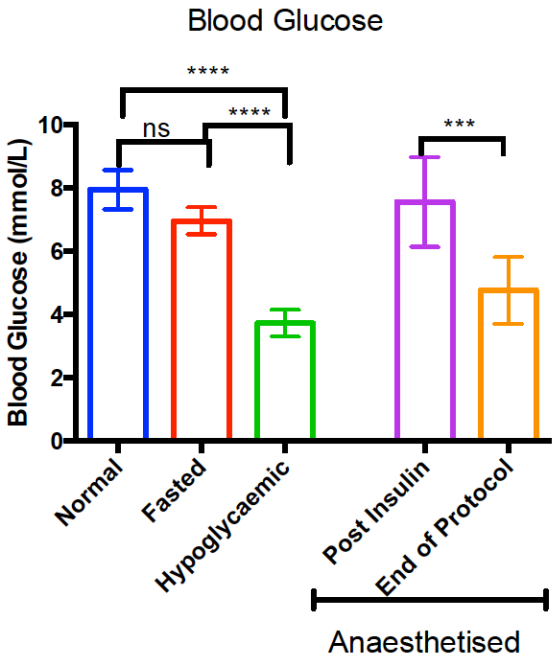

Figure 13: ***Blood glucose measurements at different stages of the protocol.*** *Mean±SD. Normal, fasted and hypoglycaemic (N=7) BG readings were compared using a repeated measure one-way ANVOA with Bonferroni's multiple comparison. Anaesthetised post insulin and end of protocol compared with a Student's paired t-test (N=6). ****P≤0.0001, ***P≤0.001.*

Table 2 shows the change in weight over the course of the protocol. As expected, animals grew normally throughout the 6week study. Blood glucose was not significantly reduced from normal by overnight fasting (P=0.0609) but dosing with

insulin (1.5IUKg^{-1}) did evoke a significant hypoglycaemia compared to both normal (P<0.0001 and fasted (P < 0.0001) levels (Figure 13).

3.3 Plethysmography studies

3.3.1 Acclimatisation

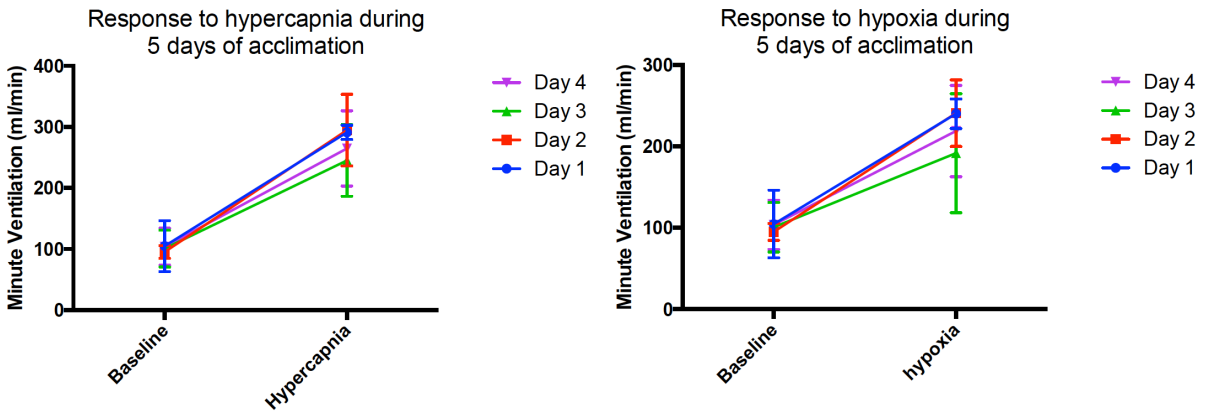

Figure 14: ***V_E at baseline and during both hypercapnia and hypoxia over 5days of acclimatisation****. Mean± SD (N=4) of baseline and responses compared with a repeated measures one-way ANOVA with Bonferroni's multiple comparison.*

A sub group of 4 rats were given 5days of acclimatisation in the plethysmography chamber. There was no change in baseline (P=0.9710) or hypercapnic minute ventilation (P=0.3962), over the 5days of acclimatisation (Figure 14). Hypoxic responses were also not significantly changed (P=0.4602). This data allowed the reduction in the number of exposures needed in the second cohort of rats (r5-8) since there was no significant effect of acclimatisation.

3.3.2. The effect of order of exposures on baseline and responses

During the protocol animals were exposed to serial exposures, although the protocol intended to return to baseline levels, to check if this was the case a standard t-test was conducted to see if there was any difference between baseline before and after the first exposure.

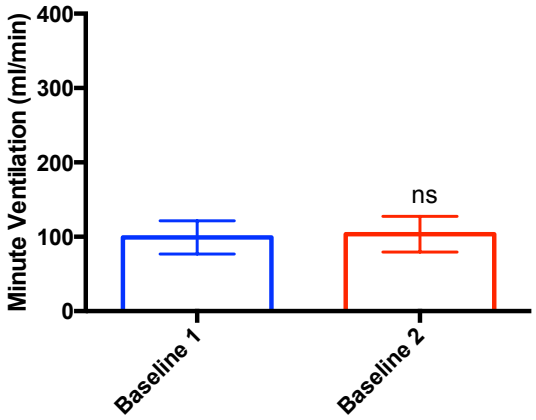

Figure 15: ***Comparison of baselines before and after the first exposure.*** *Mean±SD (N=8) compared with a Student's paired t-test.*

Baseline V_E before exposure 1 or 2 was not significantly different (P=0.15 figure 15). This was the same for all respiratory parameters including V_T (P=0.8701) and R_f (P=0.1425; data not shown) thus showing that serial exposures did not affect subsequent baselines. There was no significant difference in second response to either hypercapnia (P=0.01) or hypoxia (P=0.33) when the order was reversed (Figure 16)

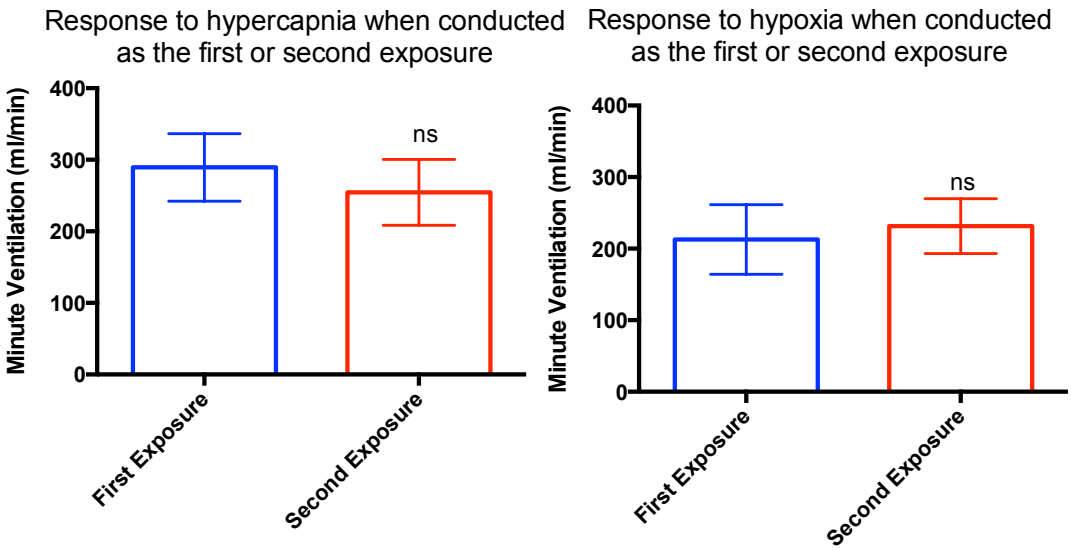

Figure 16: ***Comparison of hypercapnic and hypoxic responses when the order of exposure is changed****. Mean±SD (N=8) compared with a Student paired t-test.*

3.3.2 Change in Ventilation During Exposures in Conscious Rats

Hypercapnia

Figure 17 shows both amplitude and frequency is increased during hypercapnia in all glycaemic levels. Figure 18 shows V_E, V_T and R_f were significantly increased during hypercapnia ($P<0.0001$) in normoglycaemia. T_i ($P=0.001$), T_e ($P=0.00075$) and end expiratory pause ($P=0.0005$) were significantly reduced during hypercapnia.

After overnight fasting the response to hypercapnia was maintained with V_E, V_T and R_f significantly increased ($P<0.0001$), T_i ($P=0.0075$), T_e ($P=0.001$) and end expiratory pause ($P=0.0038$) response was also maintained. Fasting had little effect on ventilatory variables at baseline or during hypercapnia; T_e however was increased compared to normoglycaemia ($P=0.0154$).

Insulin induced hypoglycaemia caused a raised response to hypercapnia. V_E ($P<0.0001$), V_T ($P<0.0001$) and R_f ($P<0.0001$) were significantly increased during hypercapnia. T_i ($P=0.01$) and T_e ($P=0.0002$) were reduced during hypercapnia to coincide with the increase in frequency. End expiratory pause was not significantly decreased during hypercapnia ($P=0.8358$). Baseline ($P=0.0228$) and hypercapnic ($P=0.0012$) V_T were significantly increased in hypoglycaemia. Hypercapnic V_E was significantly increased in Hypoglycaemia ($P=0.0236$). In fasted testing T_e was significantly increased ($P=0.0273$) at baseline compared to normoglycaemia. Despite a clear reduction in baseline EEP in hypoglycaemia (Figure 18) there was no statistical difference between glycaemic levels.

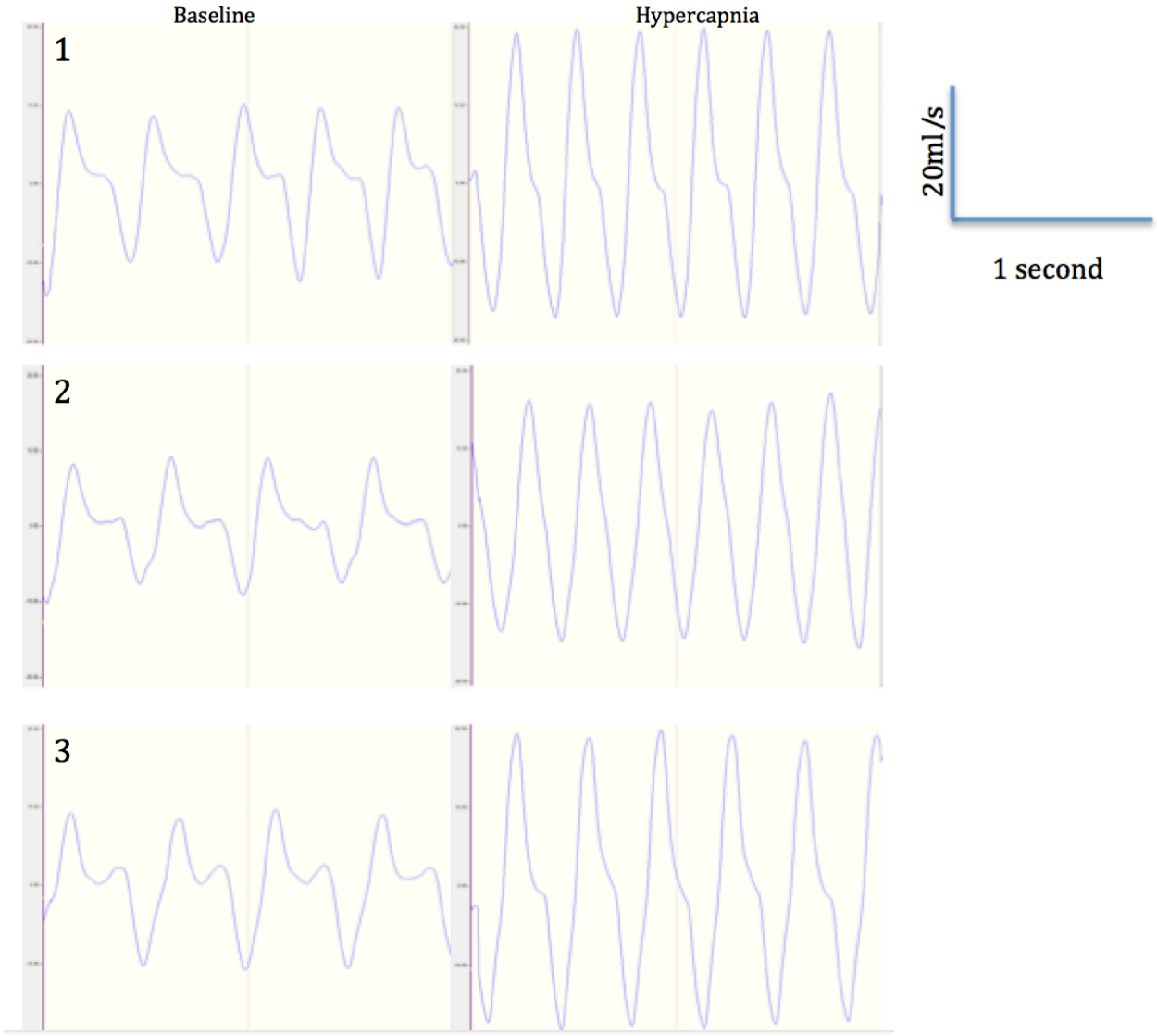

Figure 17: ***Example traces at baseline and hypercapnic response during plethysmography experimentation at different glycaemic levels****. 1. Normoglycaemic response. 2. Fasted responses 3.Insulin-induced hypoglycaemic responses.*

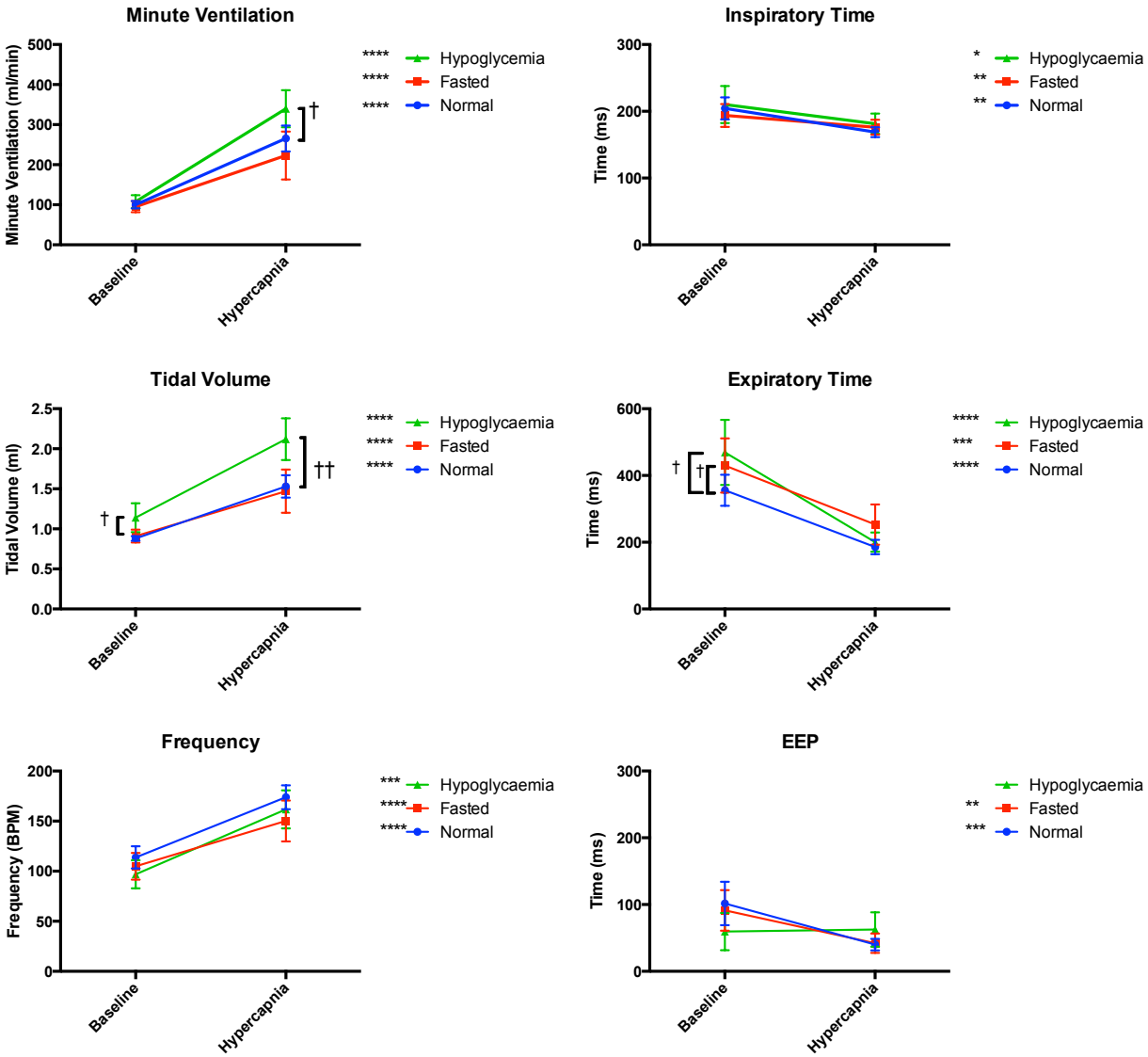

Figure 18: ***Change in ventilatory variables during hypercapnia at different glycaemic levels****, normal (blue), fasted (red) and hypoglycaemic (green). Mean±SD of baseline and hypercapnic responses compared with a Student's paired t-test. Differences between baseline and responses in normoglycaemic (N=8), fasted (N=7) and hypoglycaemic (N=7) compared with a repeated measures one-way ANOVA. ****P≤0.0001, ***P≤0.001, **P≤0.01, *P≤0.05 and ns P>0.05. ††P≤0.01, †P≤0.05*

Hypoxia

Hypoxia caused an increase in both frequency and amplitude in all glycaemic levels (Figure 19). Figure 20 shows V_E, V_T and R_f were significantly increased in response to hypoxia in normoglycaemia (P=0.0001). The increase in R_f coincided with the decrease in T_i (P<0.0001), T_e (P=0.003) and EEP (P=0.0004) in hypoxia.

Overnight fasting, as in hypercapnia, had little effect on the response to hypoxia. V_E ($P<0.0001$), V_T ($P<0.0001$) and R_f ($P=0.0014$) were still increased during hypoxia. T_e ($P=0.0105$) and EEP ($P=0.0024$) were also significantly reduced however there was no statistical decrease in T_i ($P=0.0623$). Hypoxic T_e was also significantly higher in fasted than normoglycaemia ($P=0.0112$).

Hypoxia caused a significant increase in V_E ($P=0.005$), V_T ($P=0.0010$) and R_f ($P=0.0005$) in insulin-induced hypoglycaemia. T_i ($P=0.0421$) and T_e ($P=0.0026$) were significantly decreased however, there was no significant decrease in EEP during hypoxia in hypoglycaemia ($P=0.2393$).

Insulin induced hypoglycaemia caused a significant increase in baseline V_T ($P=0.02$) as with hypercapnia. Unexpectedly it also significantly reduced respiratory frequency at baseline ($P=0.0134$) and during hypoxia ($P=0.0058$) although this was only a very slight decrease of 20BPM it is still possible to make out on the example traces in figure 19 and counter acts the significant increase in V_T. This decrease in frequency comes from an increase in T_e at baseline ($P=0.0137$) and hypoxia ($P=0.0285$).

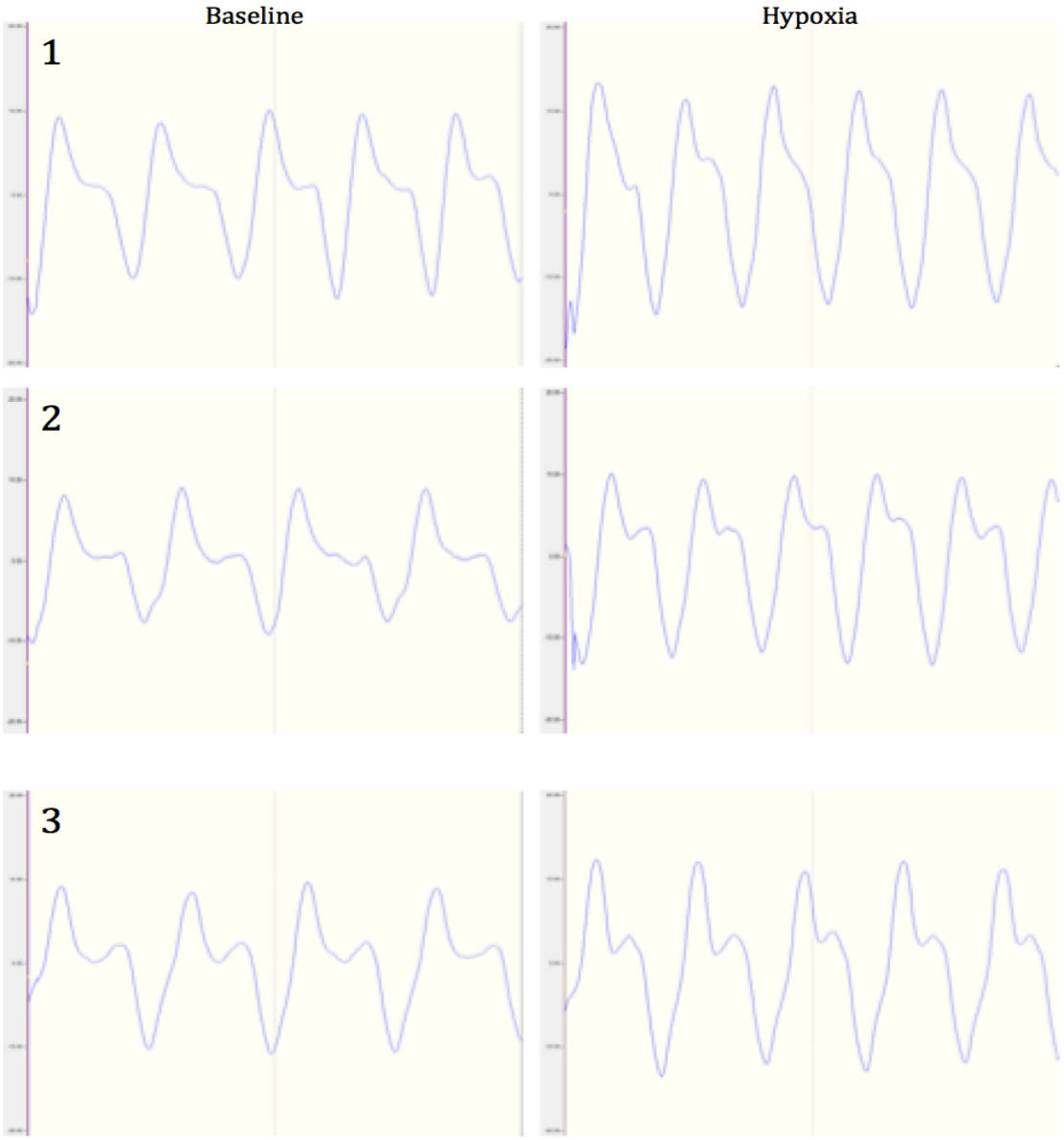

Figure 19: ***Example traces at baseline and hypoxic response during plethysmography experimentation at different glycaemic levels****. 1. Normoglycaemic response. 2. Fasted responses 3.Insulin induced hypoglycaemic responses.*

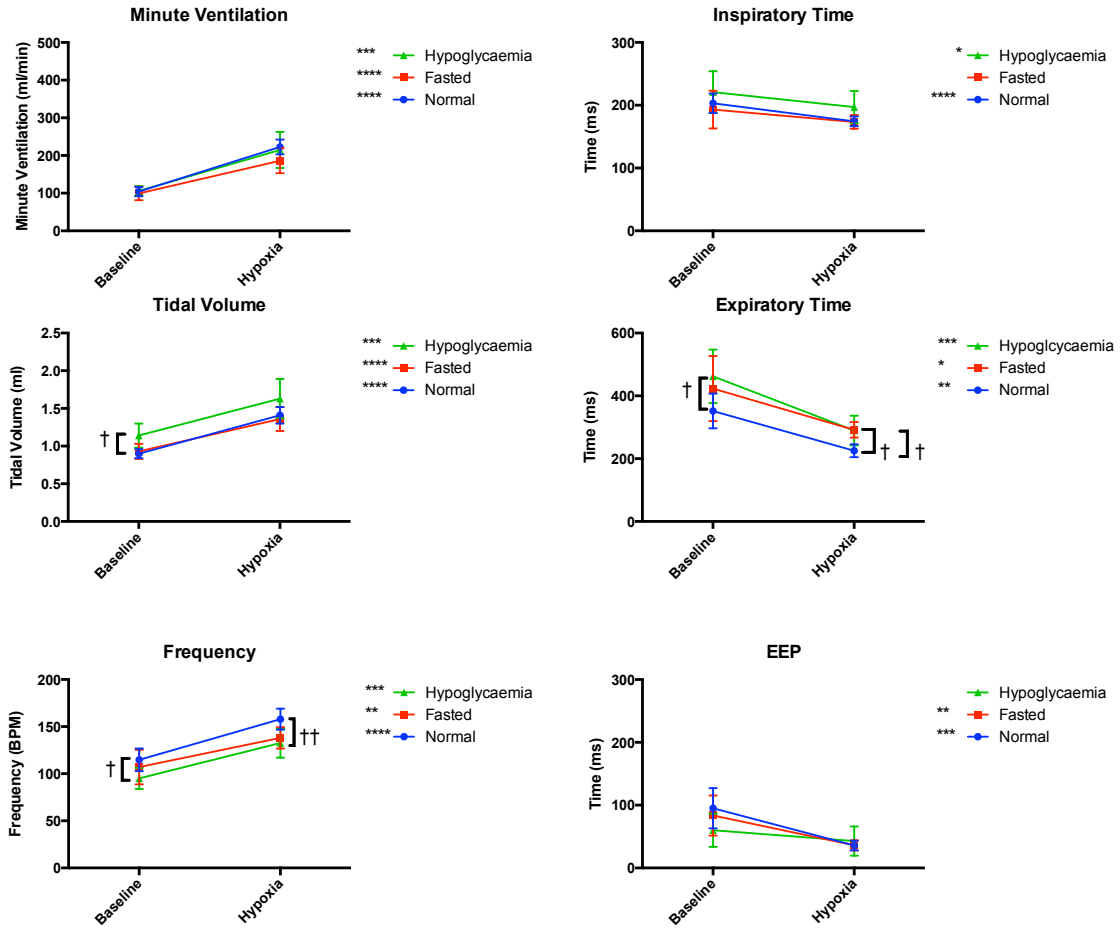

Figure 20: ***Change in ventilatory variables during hypoxia at different glycaemic levels****, normal (blue), fasted (red) and hypoglycaemic (green). Mean±SD of baseline and hypoxic responses compared with a Student's paired t-test. Differences between baseline and responses in normoglycaemic (N=8), fasted (N=7) and hypoglycaemic (N=7) compared with a repeated measures one-way ANOVA. ****P≤0.0001, ***P≤0.001, **P≤0.01, *P≤0.05 and nsP>0.05. ††P≤0.01, †P≤0.05*

Hyperoxia

Hyperoxia only induced a significant change in V_E during hypoglycaemia (P<0.001). In both normal and fasted states there was no significant change. This variation in response is also shown in the results for respiratory frequency, which is significantly increased in both normal (P=0.0192) and fasted (P=0.0003) readings but significantly decreased during hypoglycaemia (P=0.0051). Tidal volume was not significantly changed at any glycaemic level.

Inspiratory time was significantly reduced in both normoglycaemic (P=0.0007) and fasted (P=0.0379) readings however, there was no significant change in T_i in insulin-induced hypoglycaemia (P=0.5277), which is reflected in figure 21 with only a very slight increase visible. Although T_e is shown to decrease in both normal and fasted readings on figure 17 it only reached significance in fasted readings ($P < 0.0001$). T_e during hypoglycaemia does not significantly change however there is a general increase.

EEP is only significantly changed in normal readings with a significant decrease (P=0.0009) during hyperoxia. When looking at figure 21 the lack in significant change in fasted is clearly shown however, during hypoglycaemia there seems to be a general trend of increase in EEP during hyperoxia.

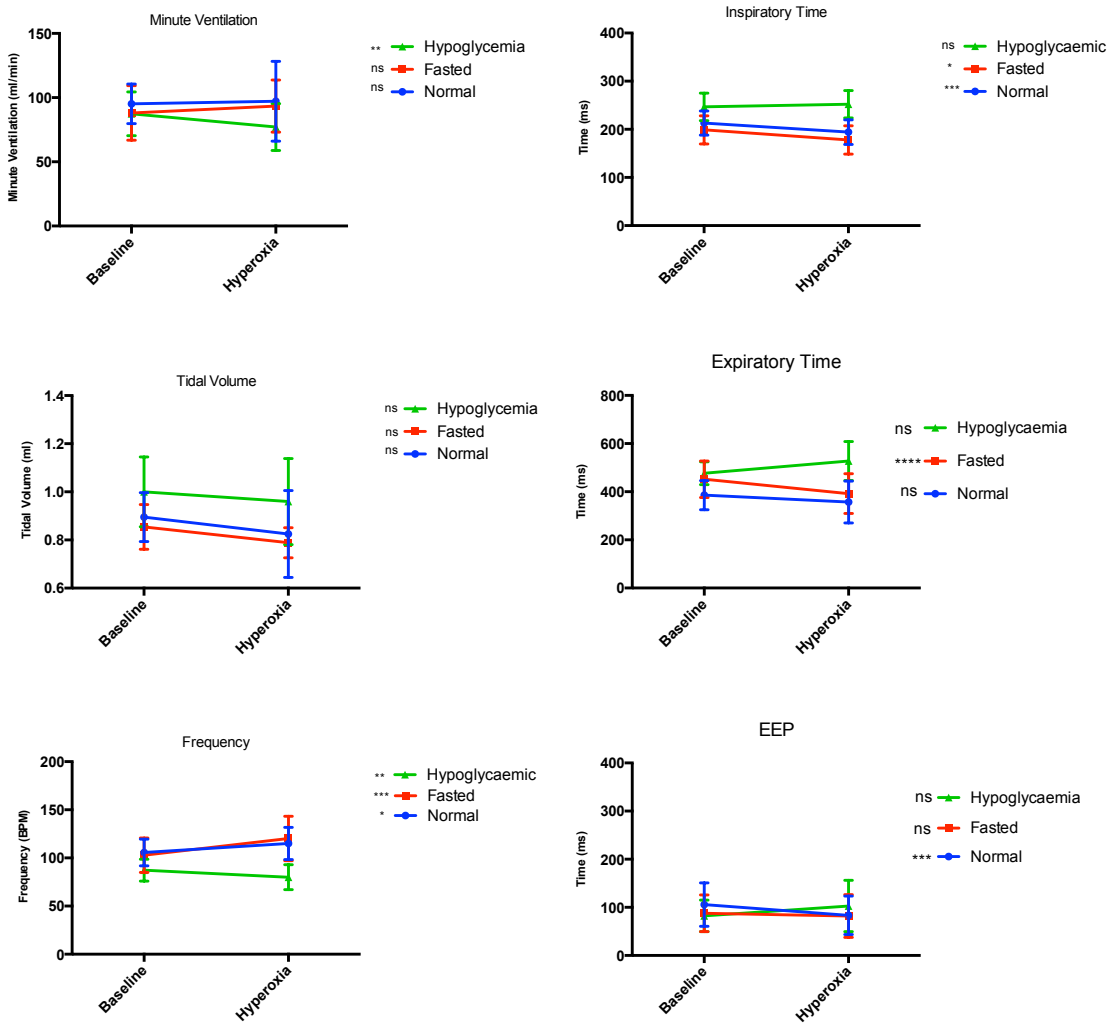

Figure 21: ***Change in ventilatory variables during hyperoxia at different glycaemic levels****, normal (blue), fasted (red) and hypoglycaemic (green). Mean±SD of baseline and hyperoxic responses compared with a Student's paired t-test. Differences between baseline and response in normoglycaemic (N=8), fasted (N=7) and hypoglycaemic (N=7) compared with a repeated measures one-way ANOVA. ****P≤0.0001, ***P≤0.001, **P≤0.01, *P≤0.05 and nsP>0.05*

During the short exposure to hyperoxia there was interference due to the movement of the rats in response to the inflow of air. As can be seen in an example trace in figure 22 there is a distortion of the normal respiratory trace that causes an increase in the frequency of peaks.

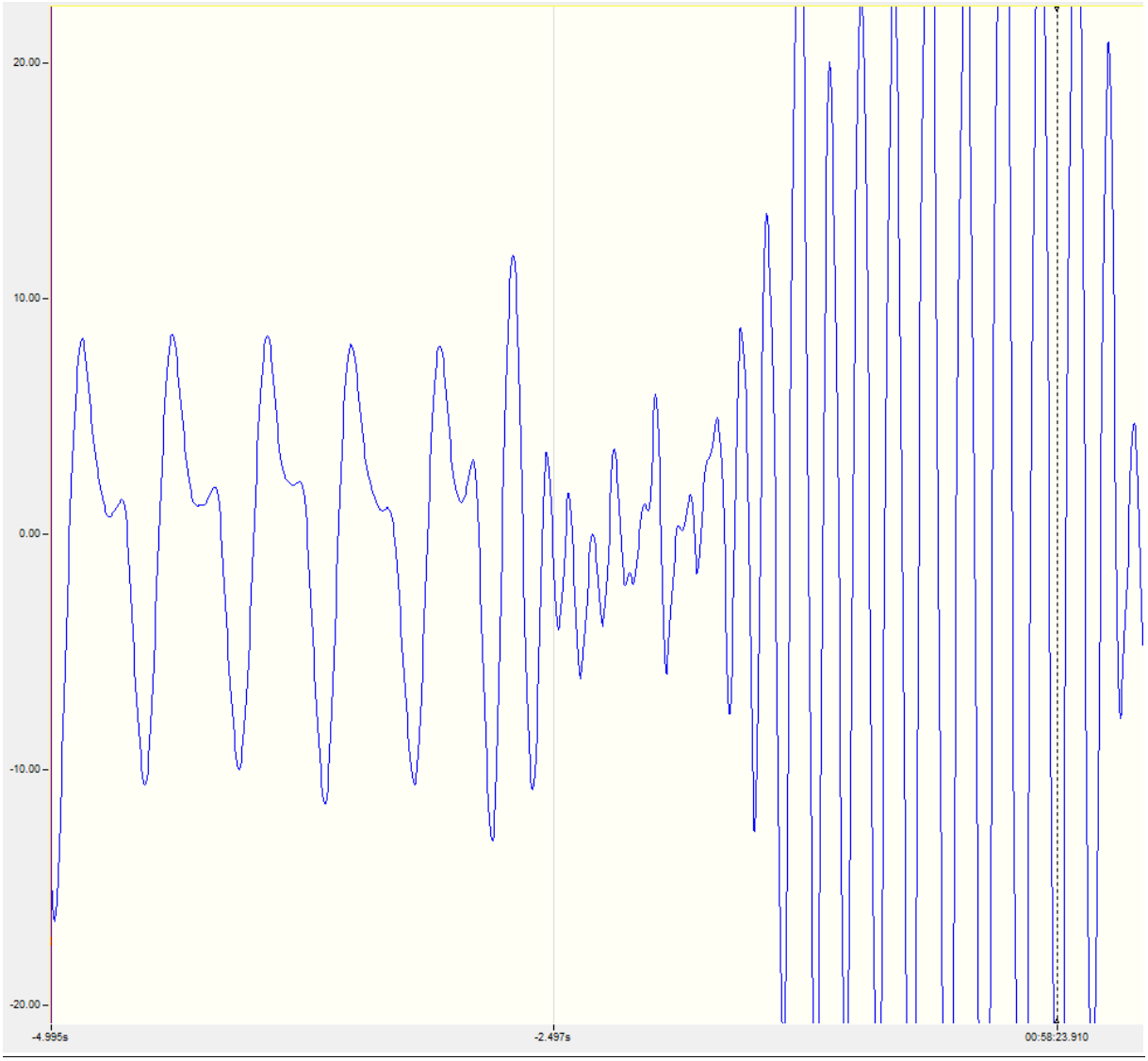

Figure 22: ***Original trace showing example of effect of animal movement caused by the onset of gas into the chamber****. Gas input was started at -2.497s.*

3.3.3 T_i:T_e ratio changes during Hypercapnia and Hypoxia

Figure 22 shows how exposure to hypercapnia and hypoxia affects the ratio of inspiratory time (T_i) to expiratory time (T_e) in conscious freely moving rats. There was a significant increase in T_i:T_e in both hypercapnia ($P<0.0001$) and hypoxia ($P=0.0007$) showing that T_e is reduced to a greater extent than T_i. When the change in T_i:T_e during hypoxia and hypercapnia were compared with a standard paired t-test

it revealed that the increase in hypercapnia was significantly higher than that found during the hypoxic response (P=0.0087). This again shows that there is a greater reduction in T_e during hypercapnia than in hypoxia.

The magnitude of the change in T_i:T_e ratio in hypercapnia is dependent on the glycaemic level. Analysis of the change by one-way repeated ANOVA shows it is significantly enhanced during hypoglycaemia compared to normal (P=0.024) and fasted (P=0.0229). The increase in T_i:T_e ratio during hypoxia, however, was not significantly affected by glycaemic level.

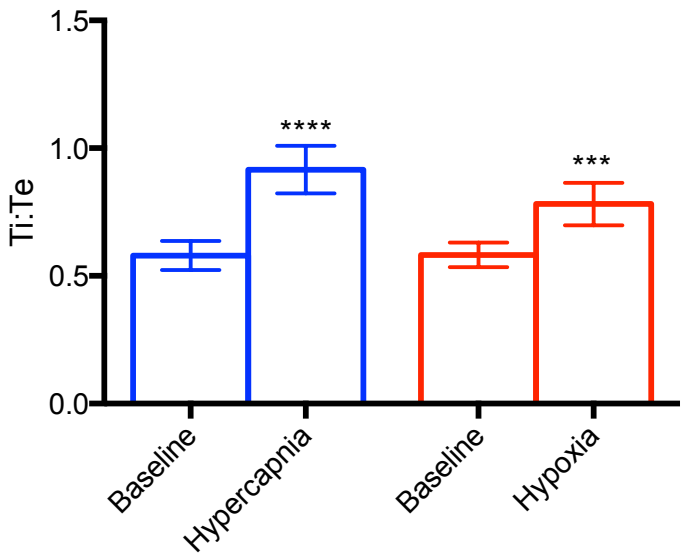

Figure 23: ***T_i:T_e during hypercapnia and hypoxia in normal glycaemic rats****. Mean±SD (N=8) baseline and responses compared with Student's paired t-test. ****P≤0.0001, ***P≤0.001*

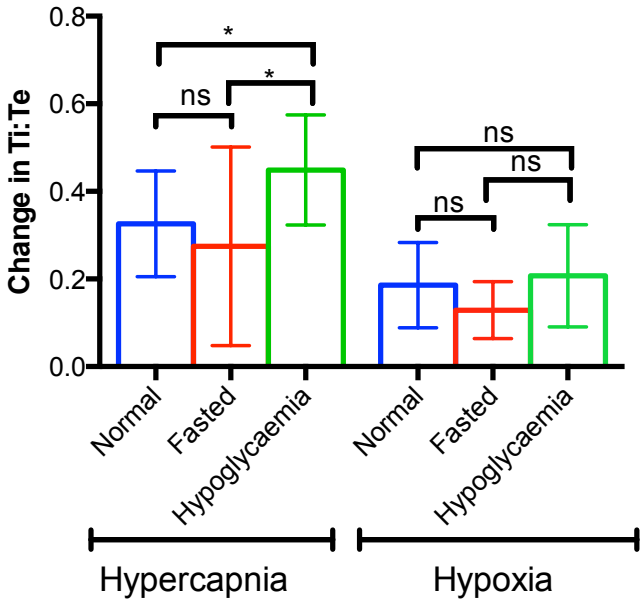

Figure 24: ***The change in T_i:T_e ratio at different glycaemic levels during hypercapnia and hypoxia****. Mean±SD (N=7) normoglycaemic, fasted and hypoglycaemic readings compared with a repeated measure one-way ANOVA with Bonferroni's multiple comparison. *P≤0.05*

3.3.4 The effect of different glycaemic levels on ventilatory variables

Hypercapnia

Blood glucose level had a significant effect on not only the response to hypercapnia but also to some baseline ventilatory variables as shown in figure 24. Regression analysis of baseline and hypercapnic minute ventilation vs. blood glucose showed that there was no correlation between the two at baseline, with the regression line not being significantly different from zero (P=0.0603) and a fit around that line of 0.1245. Hypercapnic minute ventilation however showed a negative correlation between the two, as the regression line was significantly different from zero (P=0.0077) and had a greater fit R^2=0.2353.

This negative correlation was found in tidal volume at both baseline and in response to hypercapnia. Regression analysis of baseline tidal volume showed that the line of best fit was significantly different from zero ($P<0.0001$) and the fit around the line was $R2 =0.4892$ showing that the relationship was ~50% due to the change in blood glucose. This was mirrored in hypercapnic tidal volume, which was also significantly increased from zero ($P<0.0001$) and had a similar level of fit ($R2 = 0.4383$).

Respiratory frequency was unaffected by glycaemic level with neither baseline or hypercapnic regression lines being significantly different from zero ($P=0.2128$, $P=0.3171$). The fit about these lines were also poor at baseline ($R^2=0.05689$) and hypercapnia ($R^2=0.03705$).

CO_2 sensitivity was calculated from the slope of the line graph for minute ventilation in figure 18 to give the change in minute ventilation per percentage of CO_2. Repeated one-way ANOVA showed that there was no significant change in CO_2 sensitivity from normal to fasted ($P=0.8388$) or between hypoglycaemia and normal readings (($P=0.8388$). There was however a significant increase in CO_2 sensitivity between fasted and hypoglycaemic ($P=0.0012$).

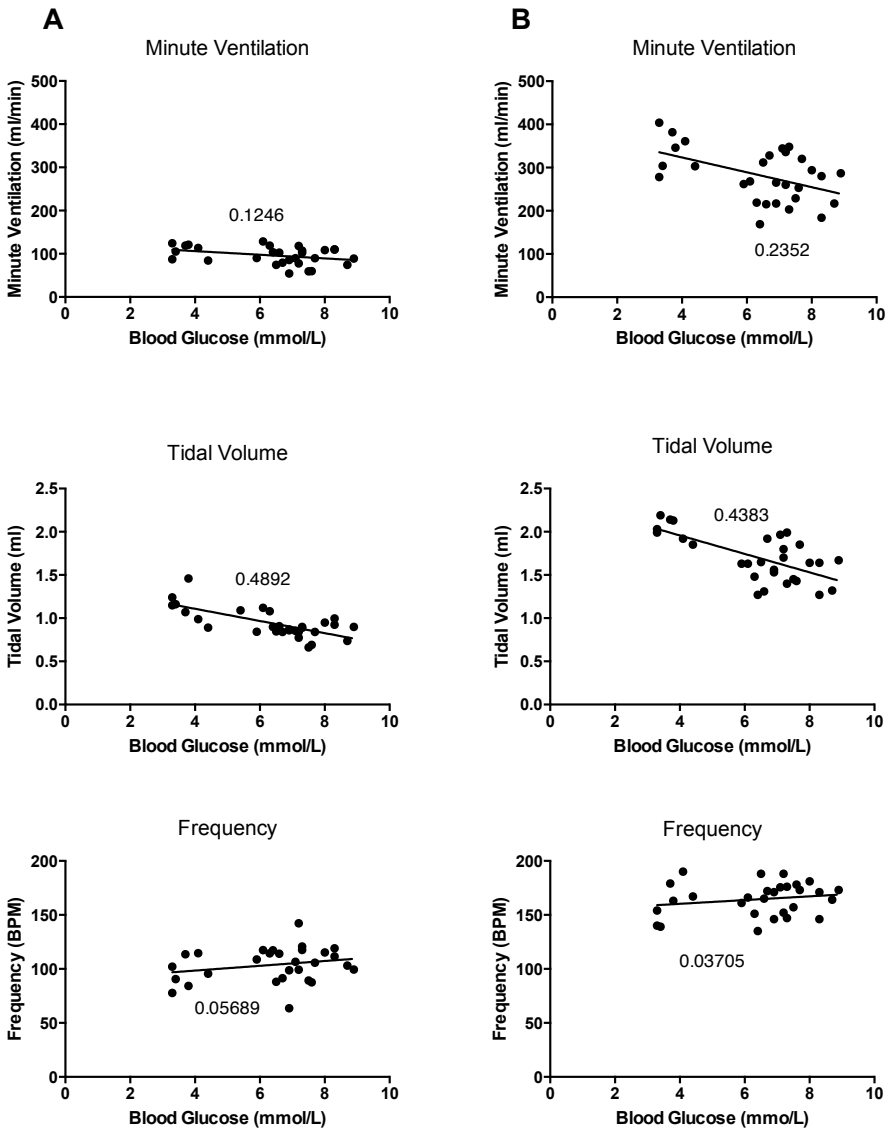

Figure 25: ***Linear regression of ventilatory variables versus BG at baseline (A) and in response to hypercapnia (B)****. Linear regressions have been plotted with* R^2 *labelled.*

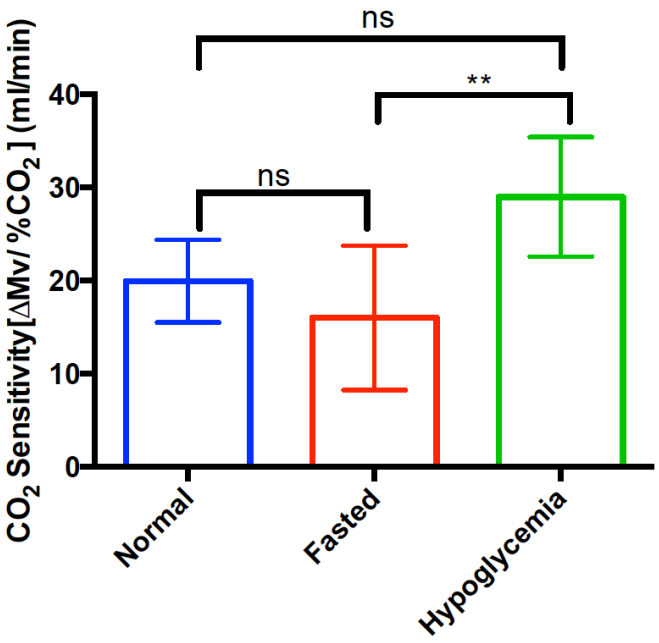

Figure 26: ***Carbon dioxide sensitivity at different glycaemic levels****. Mean±SD (N=7) sensitivity was calculated from the slope of line graphs for V_E in figure 18. Compared using a repeated measure one-way ANOVA with Bonferroni's multiple comparison. ***P≤0.001, nsP>0.05*

Hypoxia

Regression analysis found no correlation between hypoxic minute ventilation and blood glucose, with a poor fit found around the regression line (R^2 =0.00322) and a slope that was not significantly different from zero (P=0.7699).

The regression line for frequency was not significant from zero (P=0.0505) and with a fit around the regression line of R^2=0.1343 it could be possible that there is a positive correlation between respiratory frequency during hypoxia and blood glucose level.

Tidal volume was significantly negatively correlated with blood glucose with a regression line that was significantly different from zero (P=0.0318) and with a fit around the line of R^2=0.1596.

Oxygen sensitivity was unchanged during hypoglycaemia (P=0.9846). However there was an unexpected decrease in oxygen sensitivity during fasted readings (P=0.0339).

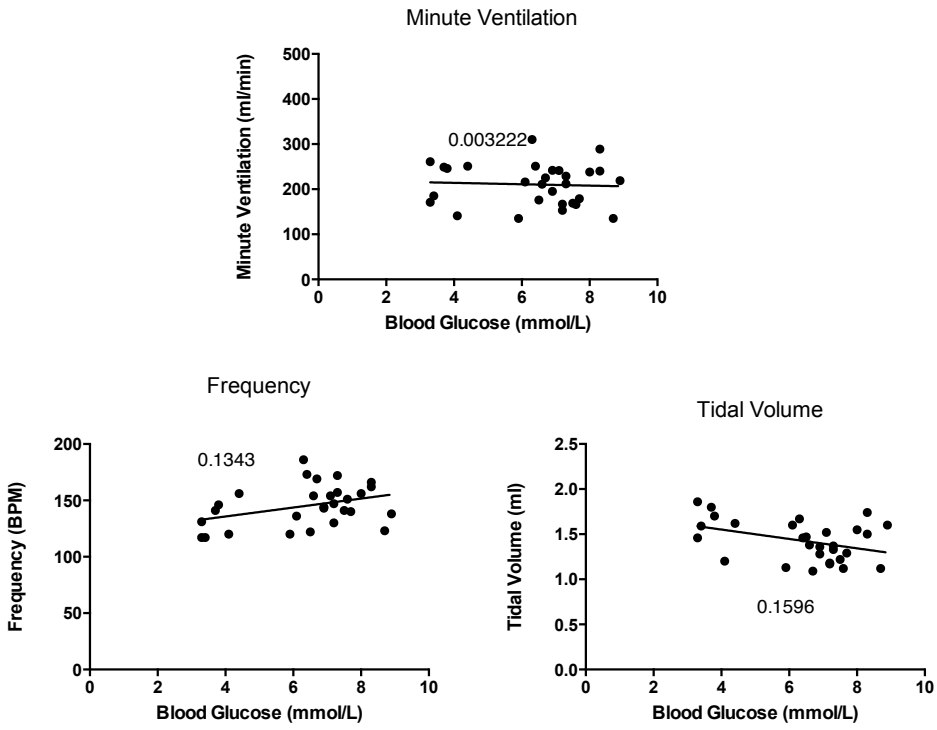

Figure 27: ***Linear regression of ventilatory variables versus BG in response to hypoxia****. Linear regressions have been plotted with R^2 labelled.*

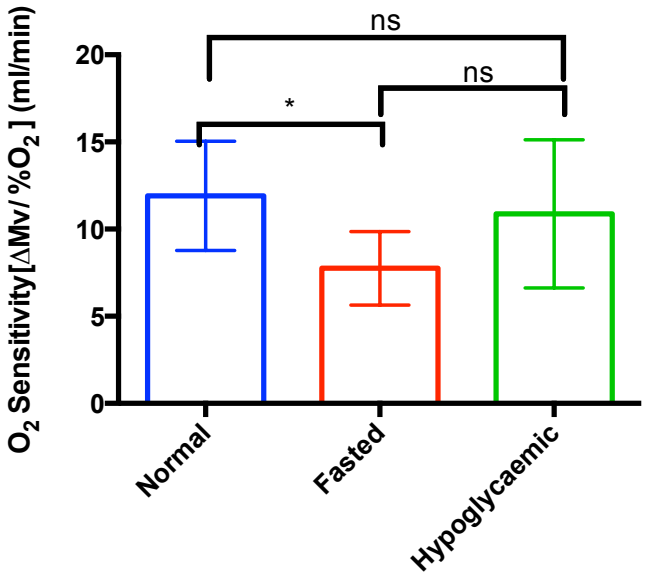

Figure 28: ***Oxygen sensitivity at different glycaemic levels****. Mean±SD (N=7) Sensitivity was calculated from the slope of line graphs for V_E in figure 20. Compared using a repeated measure one-way ANOVA with Bonferroni's multiple comparison. *P≤0.05 and nsP>0.05*

3.4 Changes in ventilation during exposures in anaesthetised rats

Hypercapnia

After the plethysmography studies the rats' response to hypercapnia, hypoxia and hyperoxia was tested under anaesthesia. Hypercapnia caused no significant increase in minute ventilation in normoglycaemic tests (P=0.0608) but did cause an increase during insulin-induced hypoglycaemia (P=0.0136). Tidal volume was significantly increased during hypercapnia in both normoglycaemia (P=0.0152) and hypoglycaemia (P=0.0202). Hypoglycaemia caused a significant rise in both baseline (P=0.0152) and hypercapnic V_T (P=0.0007) compared to normoglycaemia.

Frequency was not significantly increased at either glycaemic level (P=0.3612, P=0.1049), which is also reflected in no significant change in T_i and T_e respectively in normoglycaemia (P=0.2081, P=0.2877) and hypoglycaemia (P=0.5083, P=0.6760).

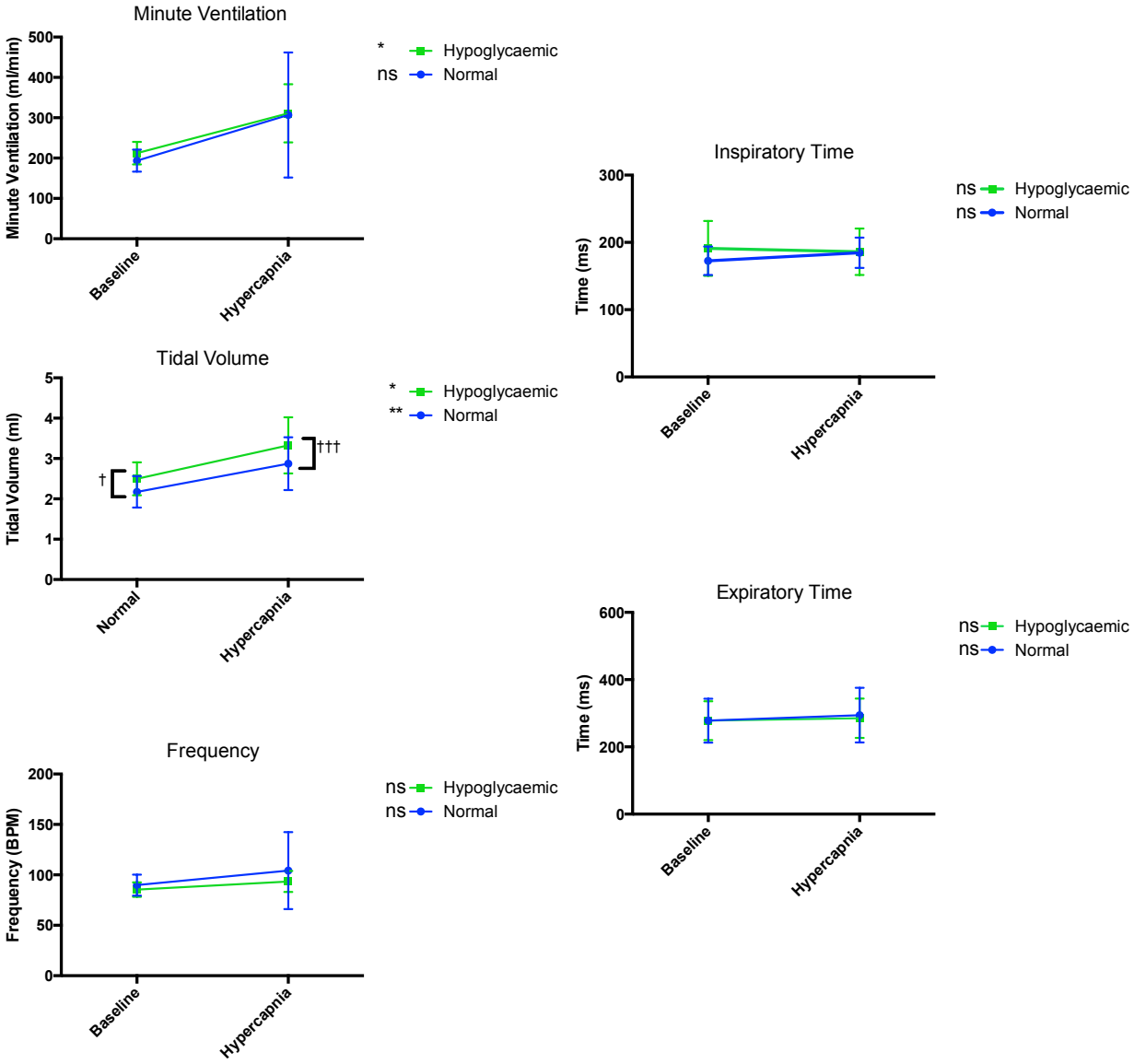

Figure 29: ***Ventilatory response to hypercapnia in anaesthetised rats****. Mean±SD of baseline and hypercapnia (N=6) and compared with a Student's paired t-test. **P≤0.01, *P≤0.05 and nsP>0.05. †P≤0.05. Difference between baseline and hypercapnic response in normoglycaemia and hypoglycaemia compared with a Student paired t-test.*

Hypoxia

The hypoxic response during anaesthesia was markedly decreased, with no significant change in minute ventilation (P=0.1674), tidal volume (P=0.1907), frequency (P=0.3019) or T_i (P=0.7642) and T_e (P=0.2357) during normoglycaemic experiments. Minute ventilation was significantly increased during hyperglycaemia (P=0.01720) however; this was not seen in other variables R_f (P=0.1007), T_i (P=0.1796), T_e (P=0.9867). There was a significant increase in hypoxic tidal volume compared to normoglycaemia in hypoglycaemia readings (P=0.0260) but this was still not enough to cause a significant change from baseline (P=0.1515).

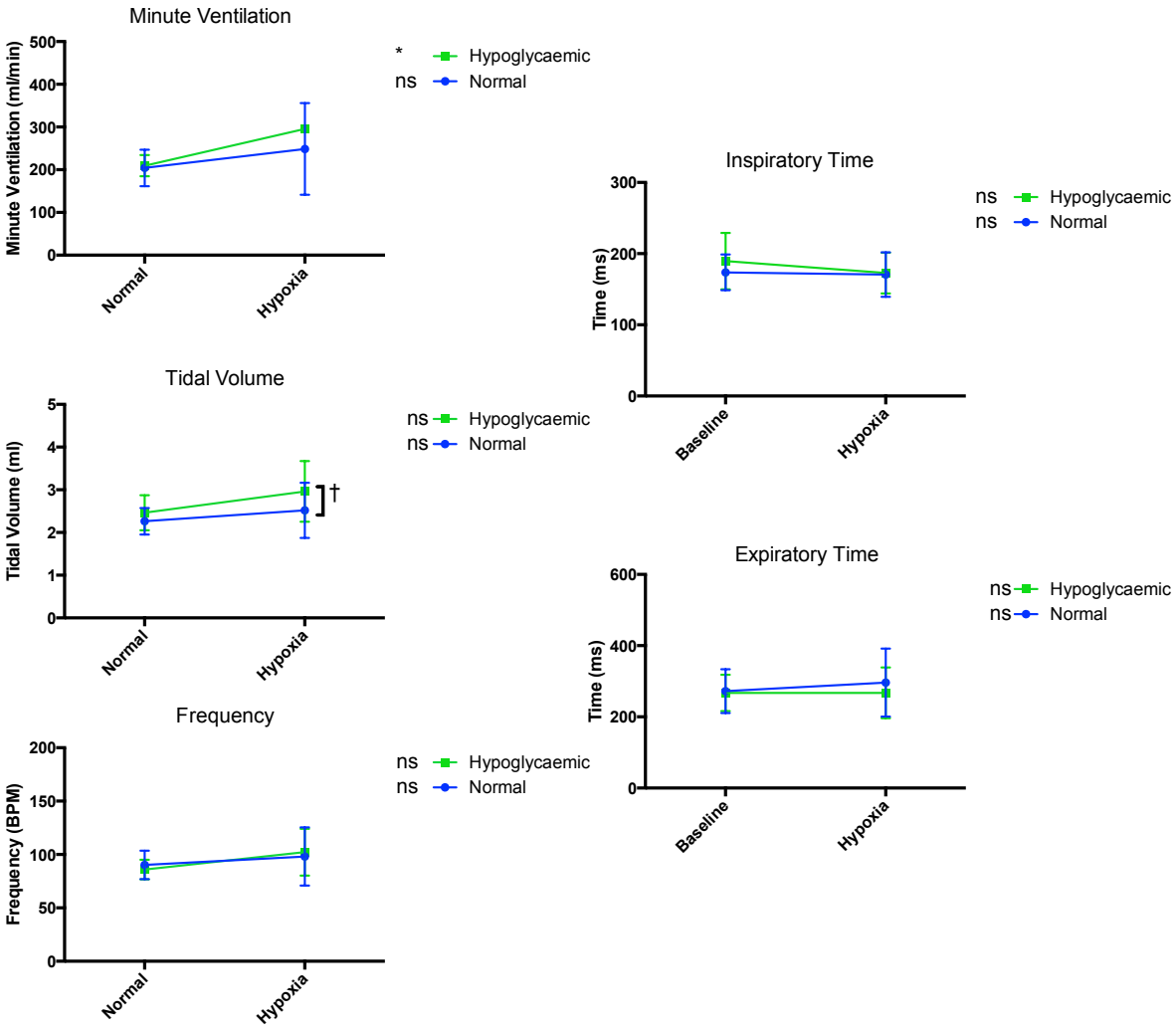

Figure 30: ***Ventilatory response to hypoxia in anaesthetised rats****. Mean±SD of baseline and hypoxia (N=6) and compared with a Student's paired t-test. **P≤0.01, *P≤0.05 and nsP>0.05. †P≤0.05. Difference between baseline and hypoxia response in normoglycaemia and hypoglycaemia compared with a Student paired t-test.*

Hyperoxia

Hyperoxia caused marked decreases in minute ventilation in anesthetised rats in normoglycaemia (P=0.0064) and hypoglycaemia (P=0.0159). This fall in minute ventilation seems to be due to a fall in respiratory frequency more than tidal volume, as frequency was significantly reduced in both glycaemic levels (P=0.0035

P=0.0171) but tidal volume was not significantly reduced in either (P=0.8647, P=0.0546). There was a slightly more pronounced drop in tidal volume during hypoglycaemia and this could be due to the significantly increased baseline tidal volume (P=0.0235). Unexpectedly there was no significant change in inspiratory (P=0.0763, P=0.6692) or expiratory time (P=0.5895, P=0.9166) at either glycaemic level, to coincide with the drop in frequency.

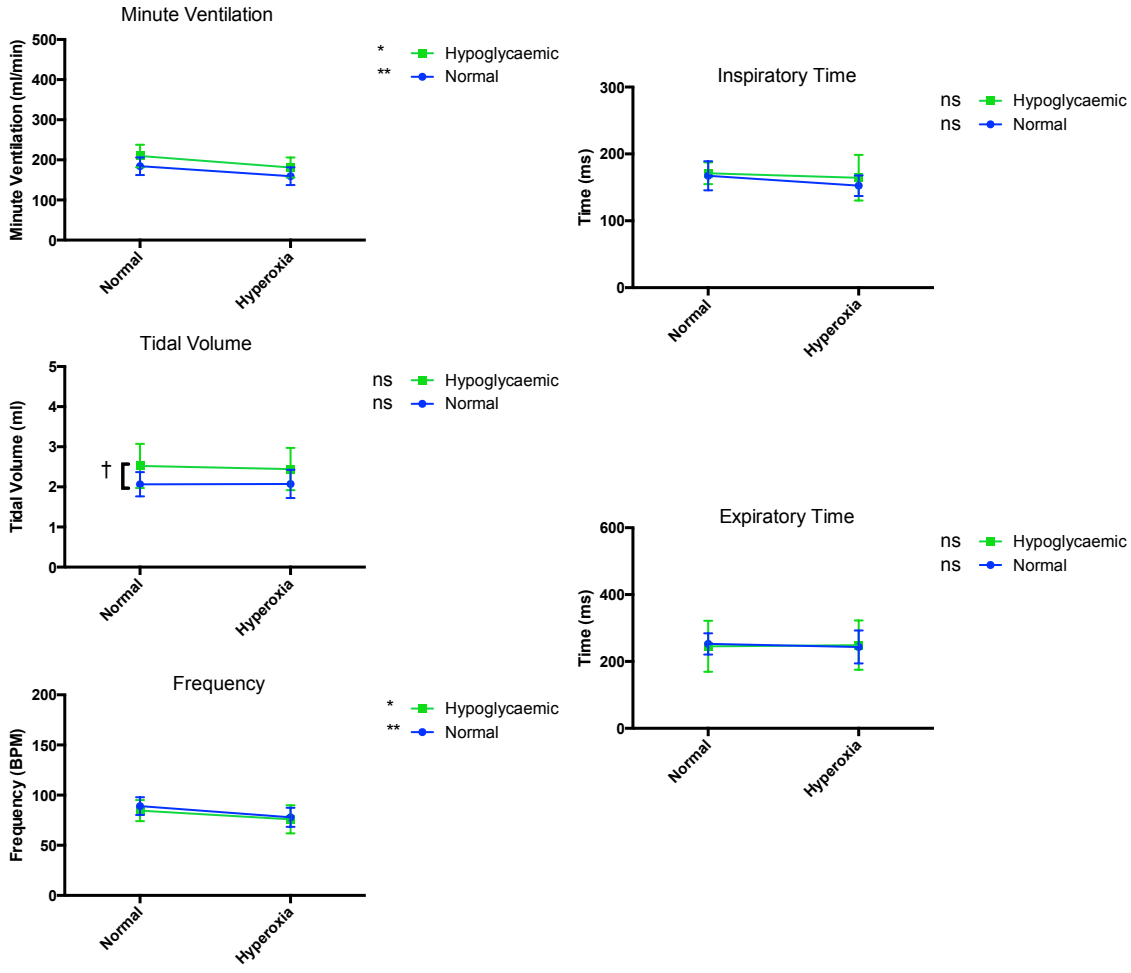

Figure 31: ***Ventilatory response to hyperoxia in anaesthetised rats****. Mean±SD of baseline and hyperoxia (N=6) and compared with a Student's paired t-test. **P≤0.01, *P≤0.05 and nsP>0.05. †P≤0.05. Difference between baseline and hyperoxic response in normoglycaemia and hypoglycaemia compared with a Student paired t-test.*

3.5 Mice studies

3.5.1 Physiological profile

Mice gained weight throughout the study but there was no significant difference between weights at any stage of the protocol. Blood glucose was not significantly changed after overnight fasting (P=0.0609). IP injection of insulin (1IUKg^{-1}) caused a significant decrease in the blood glucose (P<0.0001). This reduction in blood glucose continued through out the protocol, as end blood glucose was significantly lower than post insulin blood glucose (P=0.0135)

	Time from start of study	Weight (g)
Normal	0 days	24.0 ± 1.62
Fasted	14 days	25.8 ± 2.04
Hypoglycaemia	21days	26.1 ± 1.43

Table 3: ***Table to show the growth of mice over the length of the protocol.*** *Mean ±SD of body weight at each point in testing. Body weight was increased during the 4week protocol.*

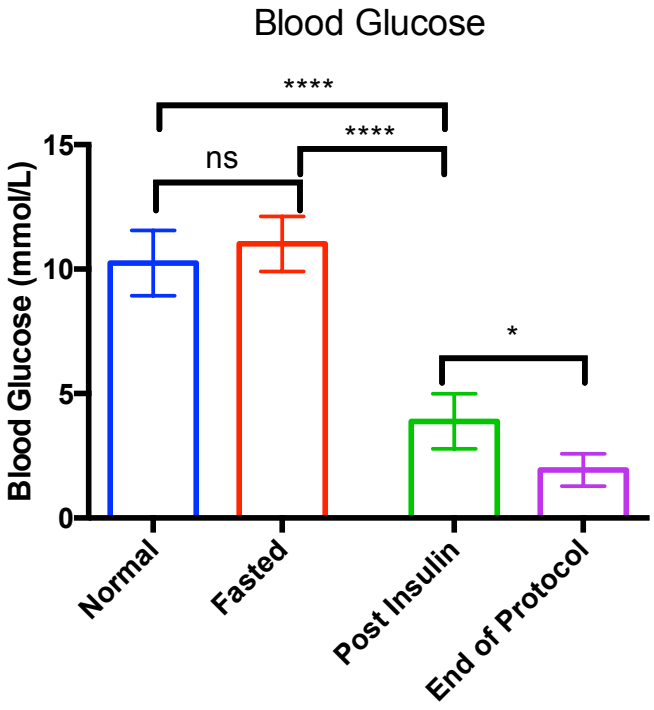

Figure 32: ***Blood glucose measurements at different stages of the protocol****. Mean±SD in normal (N=8), fasted (N=7) and post insulin (n=7) were compared with a repeated measure one-way ANOVA with Bonferroni's multiple comparison. End of protocol readings were compared to post insulin with a Student's paired t-test (N=6) ****P≤0.0001, *P≤0.05 and nsP>0.05*

3.5.2 Response to hypercapnia, hypoxia and hyperoxia in control, fasted and hypoglycaemic conditions

Hypercapnia

Hypercapnia induced a significant response in mice plethysmography studies during normoglycaemic and fasted tests (Figure 32). Minute ventilation was significantly raised during hypercapnia in both normal (P=0.0007) and fasted (P<0.0001) mice. This links with the significant increase in both tidal volume and frequency in both normal (P=0.0008,P=0.0026) and fasted (P<0.0001, P=0.0007) readings. Inspiratory time was significantly decreased in both normal (P=0.0106) and fasted (P=0.0195)

groups but to a lesser extent than the reduction seen in expiratory time, which again was reduced in both normal (P=0.0001) and fasted group (P<0.0001). End expiratory pause was also significantly reduced in normal (P=0.0029) and fasted tests (P=0.0018).

The response to hypercapnia was unexpectedly blunted in insulin-induced hypoglycaemia. Minute ventilation was increased but not to a significant level (P=0.0984) as were tidal volume (P=0.0124) and frequency (P=0.19). This coincided with a non-significant drop in T_i (P=0.1016) and T_e (P=0.5198). End expiratory pause was actually slightly increased during hypercapnia in hypoglycaemia however again this was not to a significant level (P=0.2043)

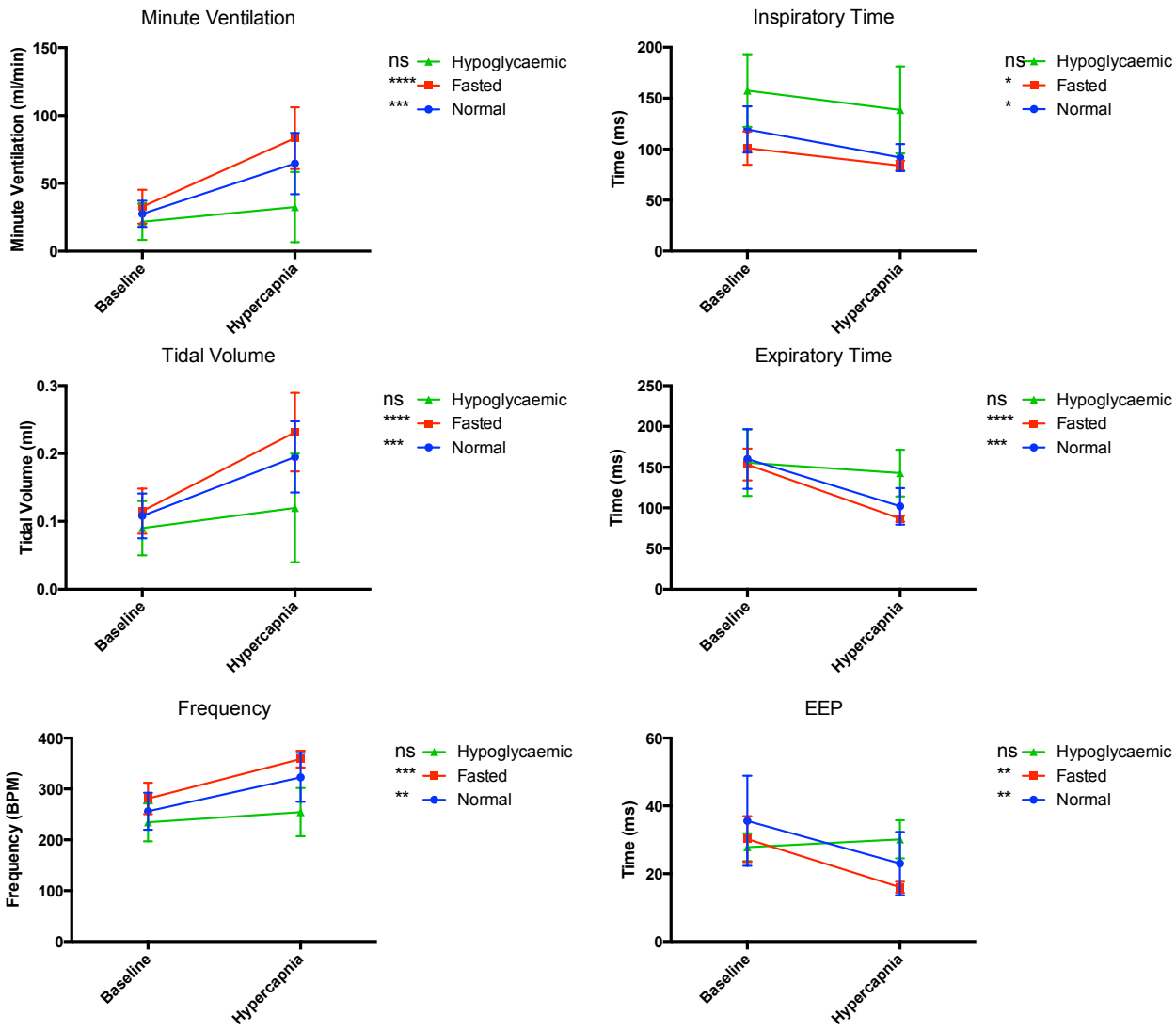

Figure 33: ***Response to hypercapnia in conscious freely moving mice at different glycaemic levels****. Mean±SD of respiratory Variables at baseline and during hypercapnia were compared with a Student's paired t-test. ****P<0.0001, ***P≤0.001, **P≤0.01, *P≤0.05 and nsP>0.05. Differences in baseline and hypercapnia values in normoglycaemia (N=8), fasted (N=7) and hypoglycaemia (N=6) were compared with a repeated measure one-way ANOVA with no significant difference found.*

When looking at the individual response to hypercapnia in hypoglycaemic mice it was clear that there was a large amount of variation in the size of response (figure 34). Mouse 2 and 5 showed a response to hypercapnia with a marked increase in V_E, V_T

and R_f which coincides with a decrease in Ti and Te. However all other mice had a depressed response to hypercapnia with no increase in V_E, V_T or R_f.

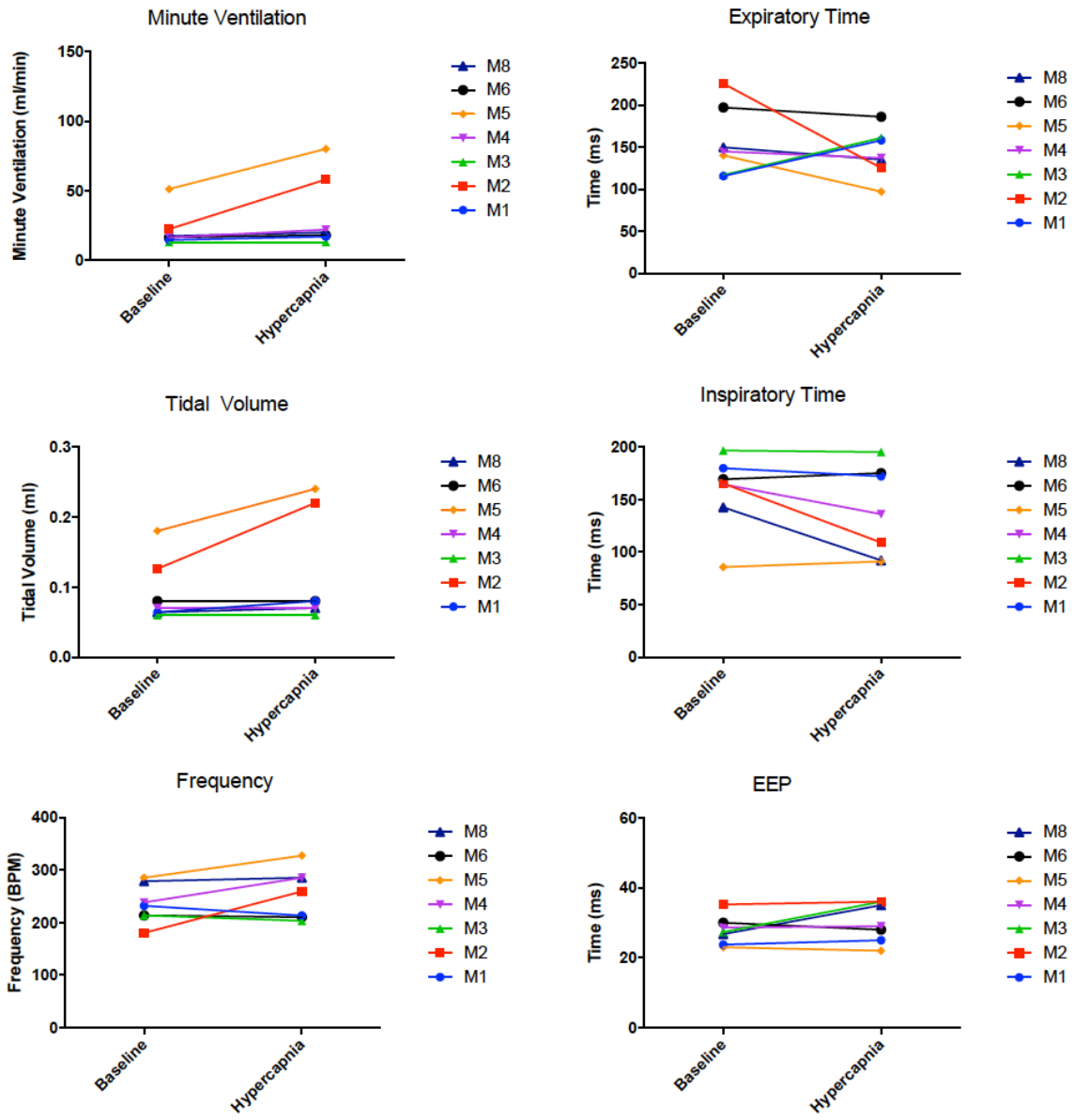

Figure 34: ***Individual response to hypercapnia in hypoglycaemic mice.***

Hypoxia

Mouse hypoxic response was very weak, as is shown in the small changes seen in figure 33. Minute ventilation was significantly increased in normoglycaemic tests only (P=0.0029) with non-significant increase being seen in fasted (P=0.4091) and no response seen in hypoglyceamic tests (P=0.7972). Tidal volume was also only increased in normal (P=0.0009) tests although both fasted (P=0.0865) and hypoglyceamic (P=0.1080) test had a trend towards an increase this was not significant.

Frequency was significantly increased in normal tests (P=0.0108) but was unexpectedly decreased in both fasted (P=0.2646) and hypoglycaemic (P=0.2929) readings although not significantly. This is matched by the decrease in T_i during hypoxia, in normal animals (P=0.0065) but increases in both fasted (P=0.3530) and hypoglycaemic (P=0.3541) readings. Expiratory time however was significantly decreased in both normal (P=0.0039) and fasted (P=0.0124) mice but had no significant decrease in hypoglycaemia (P=0.2279). End expiratory pause was significantly decreased in response to hypoxia at normoglycaemia (P=0.0033) but was not significantly decreased in fasted (P=0.2892) or hypoglycaemia (P=0.4583).

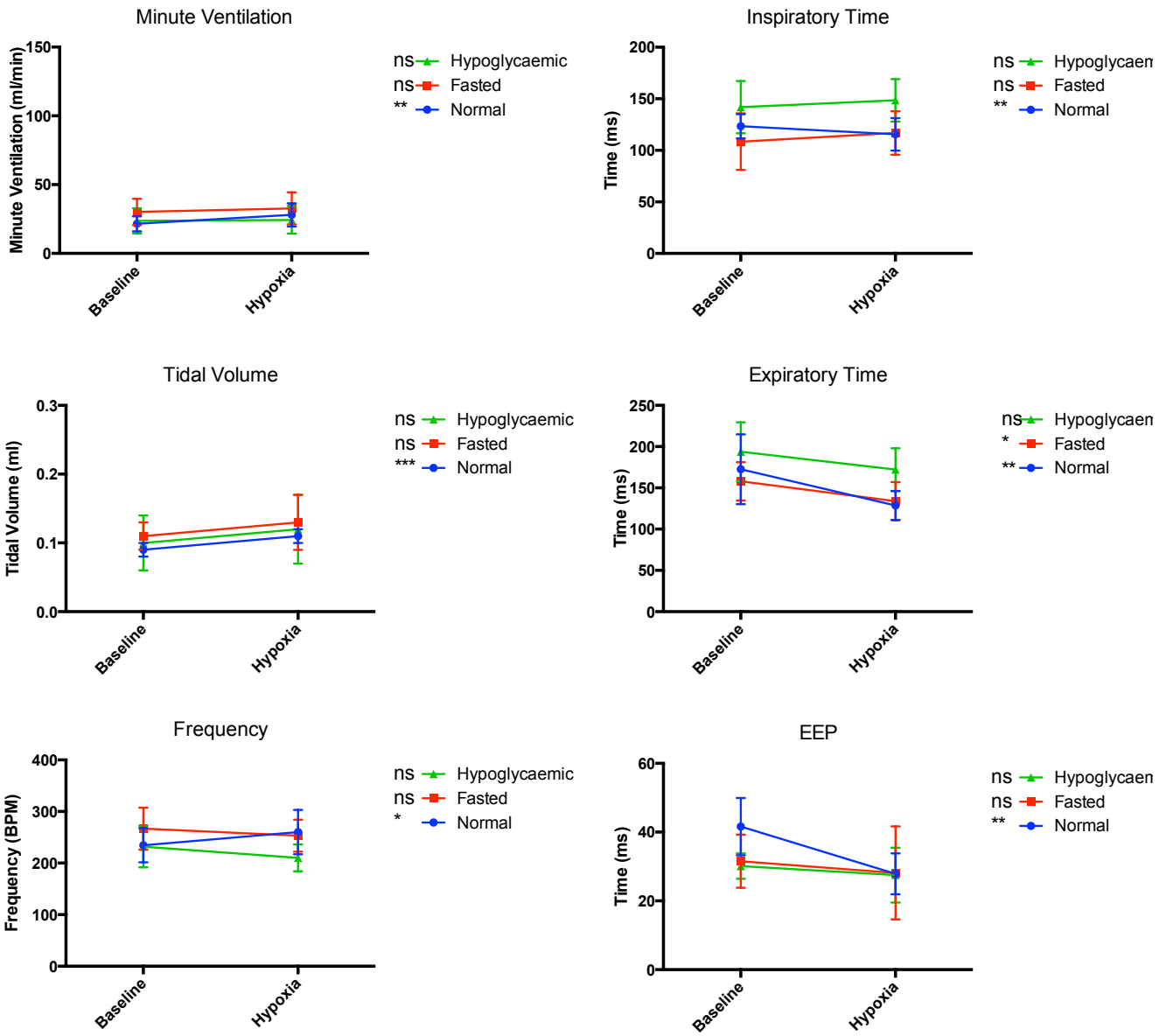

Figure 35: ***Response to hypoxia in conscious freely moving mice at different glycaemic levels****. Mean±SD respiratory variables at baseline and during hypoxia were compared with a Student's paired t-test.***P≤0.001, **P≤0.01, *P≤0.05 and nsP>0.05. Differences in baseline and hypercapnia values in normoglycaemia (N=8), fasted (N=7) and hypoglycaemia (N=6) were compared with a repeated measure one-way ANOVA with no significant difference found.*

When looking at the individual response's to hypoxia in hypoglycaemic mice there was again a variation in the responses between animals (Figure 36). There was a small increase in VE in mouse 3, 5 and 6, but in all others there is a drop in minute

ventilation during expose to hypoxia. Vt was increased in mouse 1, 3, 5 and 6 but decreased in mouse 2 and 8. Frequency was highly variable with mouse 1 and 8 showing a decrease in Rf during hypoxia, with smaller decreases being seen in mouse 2 and 6. In contrast there was an increase in Rf in mice 3 and 5. Inspiratory time was increased in mouse 1, 2, 3, 5 and 8, but decreased in mouse number 4. Expiratory time was increased in mice 1 and 8 but decreased in mouse 2, 3, 5 and 6. End expiratory pause was increased in mouse 1, 6 and 8 but decreased in all others.

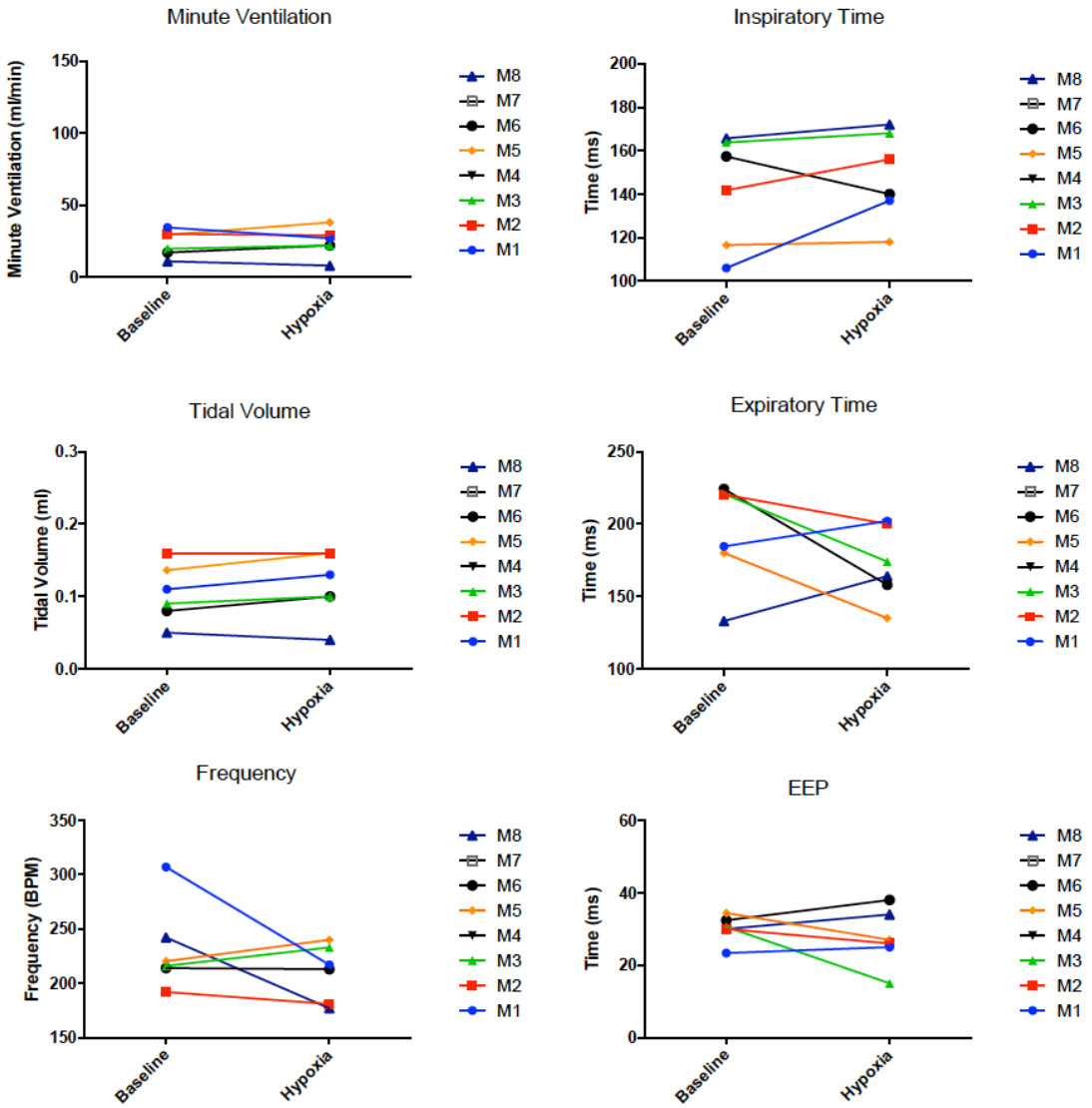

Figure 36: ***Individual response to hypoxia in hypoglycaemic mice***

Hyperoxia

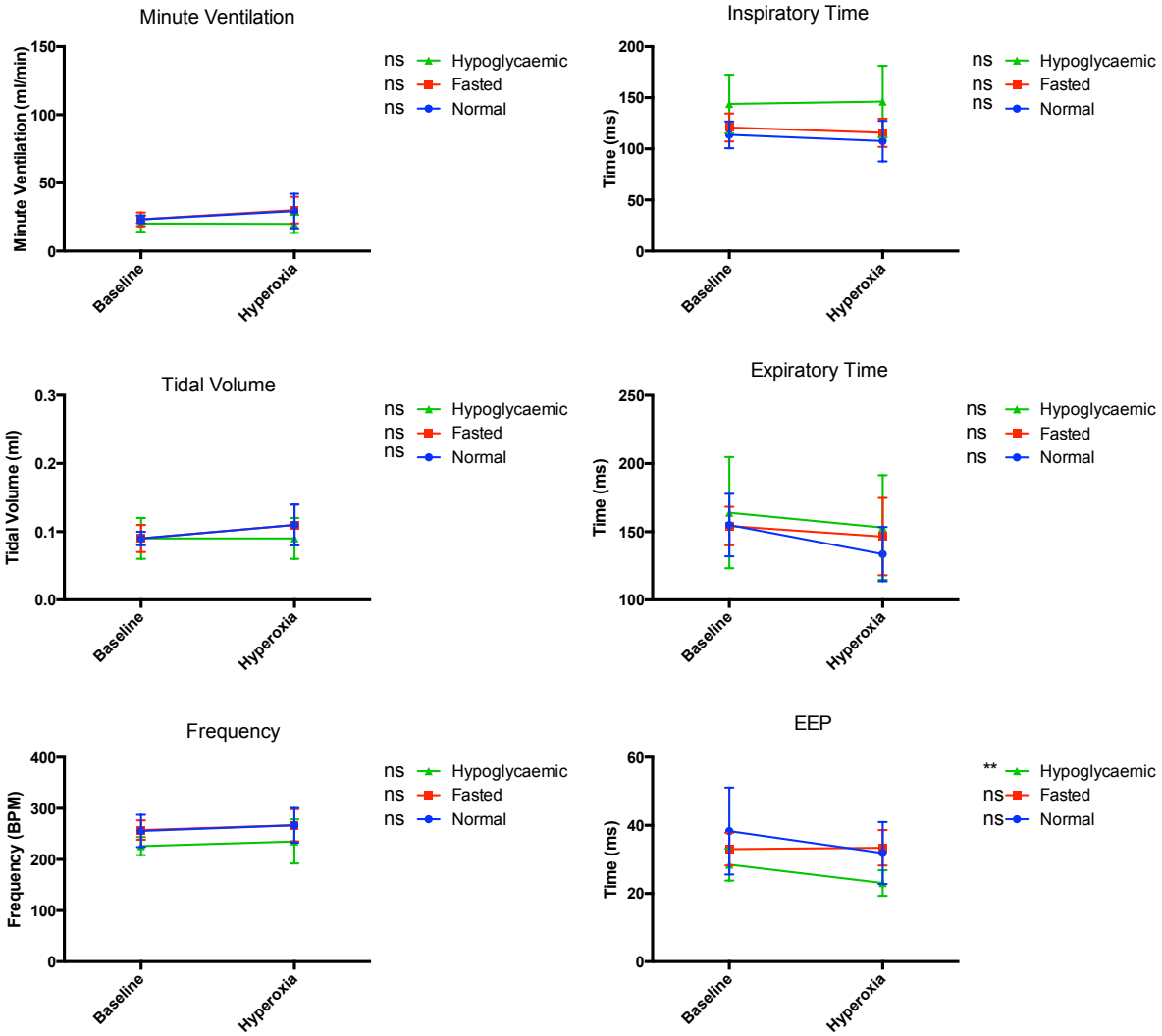

Figure 37: ***Response to hyperoxia in conscious freely moving mice at different glycaemic levels****. Mean±SD of respiratory variables at baseline and during hyperoxia were compared with a Student's paired t-test. ***P≤0.001, **P≤0.01, *P≤0.05 and nsP>0.05. Differences in baseline and hypercapnia values in normoglycaemia (N=8), fasted (N=7) and hypoglycaemia (N=6) were compared with a repeated measure one-way ANOVA with no significant difference found*

Exposure to 100% O_2 (hyperoxia) did not have the expected effect in mice. There was no significant change in minute ventilation at any glycaemic level (P=0.1936, P=0.1306, P=0.2955) however there was a small trend for V_E to increase (Figure 34).

This trend is matched by the non-significant increase in both tidal volume and frequency in normal (P=0.1255, P=0.4599), fasted (P=0.1340, P=0.3309) and hypoglycaemic (P=0.2395, P=0.5282) readings, in place of the expected decrease.

Inspiratory and expiratory times were also not significantly effected by exposure to hyperoxic gas flow at normal (P=0.4701, P=0.1509), fasted (P=0.4234, P=0.4198) or hypoglycaemic levels (P=0.6648, P=0.1322). EEP was significantly reduced in hypoglycaemia (P=0.0085) but not in normal (P=0.3107) or fasted (P=0.8994).

3.5.3 Effect of Blood glucose on Ventilatory response to Hypercapnia and Hypoxia

Hypercapnia

In the full data set blood glucose was not correlated with baseline V_E (P=0.648) with a fit around the line of regression of R^2 =0.1769. Hypercapnic V_E had a significant positive correlation with blood glucose (P=0.0005) and a very good fit with the data (R^2=0.4999) this was not what was expected or what was found in the previous rat experiments (Figure 24). Baseline tidal volume was not significantly changed by blood glucose in the full data set (P=0.1539) the fit around the line was R^2=0.1670. Hypercapnic V_T was positively correlated with blood glucose (P=0.0010) with a goodness of fit of (R^2=0.4585). Frequency showed a positive correlation at both baseline (P=0.0648) and hypoxia (P=0.0002) in the full data set.

By removing the extreme lows out the data set blood glucose was still not correlated with baseline V_E (P=0.6911) and a reduced fit around the line of regression of R^2=0.01255, as would be expected when removing points out of the data§ set.

Hypercapnic V_E was not correlated with blood glucose in reduced data set with no significant difference from zero (P=0.6777) and had a reduced fit (R^2=0.01371). Baseline V_T was like in the full data set was not correlated with blood glucose at baseline (P=0.1305) with a fit around the regression line of R^2=0.1096. Hypercapnic V_T was not correlated (P=0.8812) with goodness of fit of R^2=0.001785. Frequency Unlike V_E and V_T was not changed by removing the low data points baseline (P=0.956) and hypercapnic (P=0.1424) frequency's were not significantly different from zero there was still a trend towards a positive correlation.

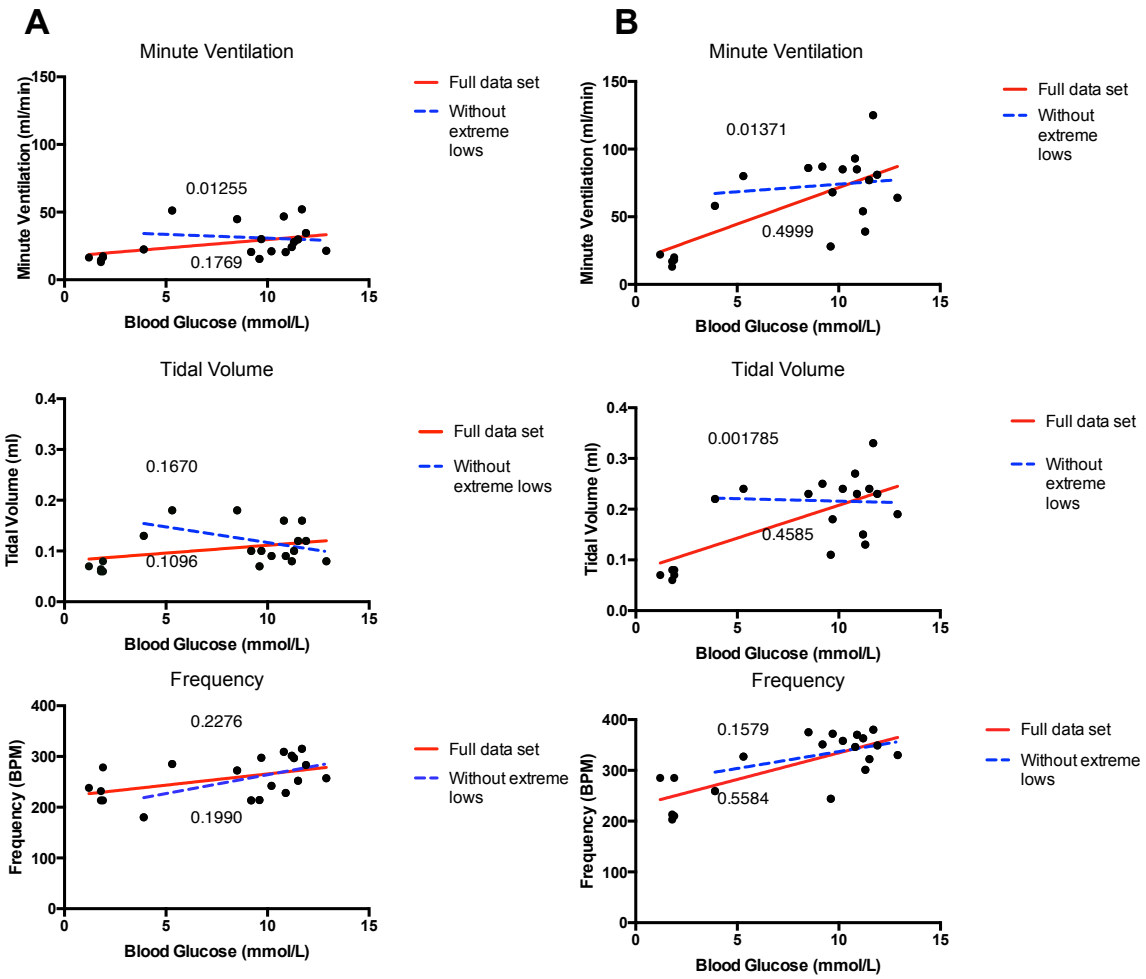

Figure 38: ***Linear regression of ventilatory variables versus BG at baseline (A) and in response to hypercapnia (B)****. Linear regressions have been plotted with* R^2 *labelled; full dataset (red) and data with BG < 2 mmolL*$^{-1}$ *excluded (blue).*

Hypoxia

Regression analysis of responses to hypercapnia (Figure 35) and hypoxia (Figure 36) revealed that correlation between blood glucose and V_E was mixed. What was apparent in many was that removal of the extremely low blood glucose readings altered the direction of correlation from positive to negative as shown by the dashed line in figures 35 & 36.

Hypoxia was largely unaffected by blood glucose with little correlation found. What was clear, as in hypercapnia the removal of the extreme lows did have a significant effect. The full data set showed by the red line in figure 36 saw no correlation between V_E and Blood glucose at baseline (P=0.3707) or during hypoxia (P=0.1311). Baseline and hypoxic V_T were also un-correlated in the fall data set. Frequency was uncorrelated at baseline (P=0.4099) but during hypoxia there was a significant positive correlation (P=0.0301).

By removing the extreme lows from the data set as represented by the blue dashed line on figure 36 V_E was still not correlated at baseline (P=0.4161) or hypoxia (P=0.3501). Baseline V_T was negatively correlated with blood glucose at baseline (P=0.0401) but this correlation was lost during hypoxia (P=0.4518). R_f was not correlated at either baseline (P=0.7812) or hypoxia (P=0.2420).

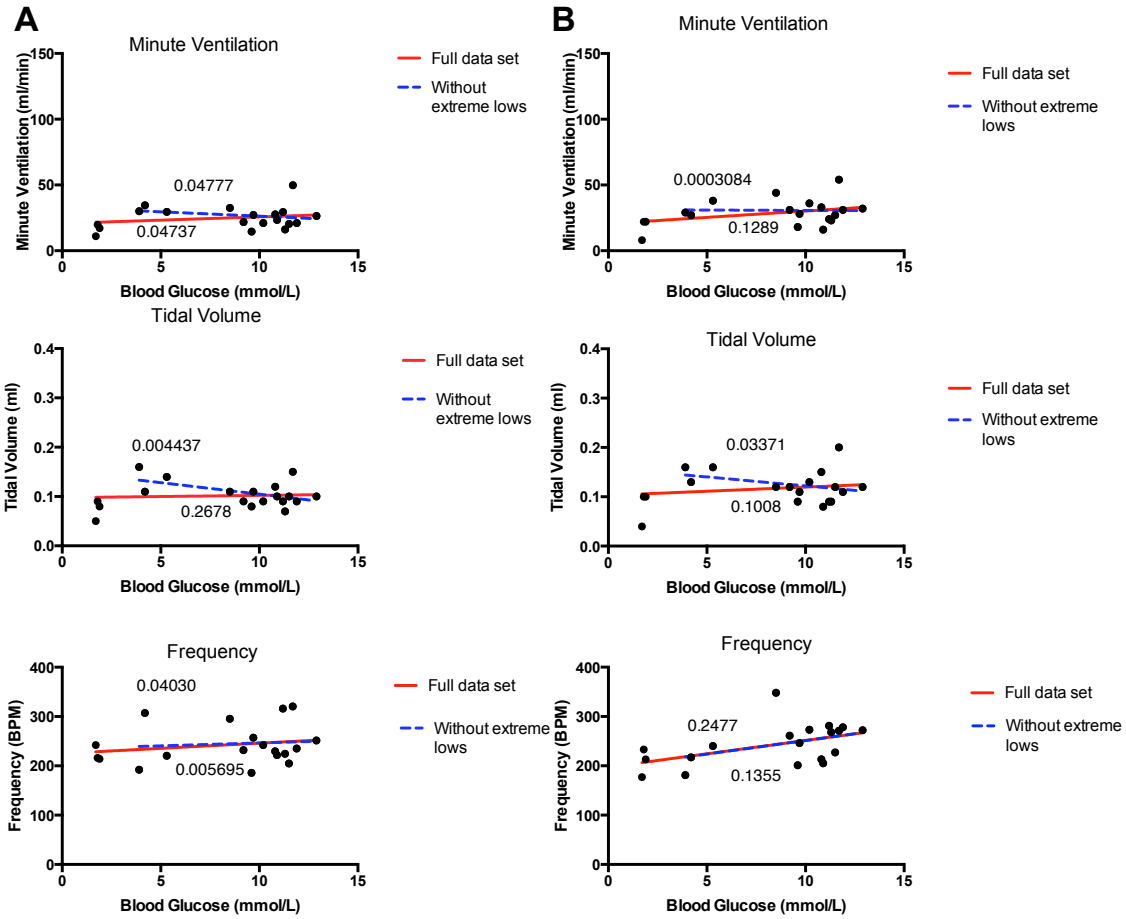

Figure 39: ***Linear regression of ventilatory variables versus BG at baseline (A) and in response to hypoxia (B)****. Linear regressions have been plotted with* R^2 *labelled; full dataset (red) and data with BG < 2 mmolL*$^{-1}$ *excluded (blue).*

4.Discussion

The aim of this study was to deduce the effect that hypoglycaemia has on the ventilatory pattern response to hypercapnia, hypoxia and hyperoxia in conscious rats and mice. Anaesthesia was also investigated for its effect on these responses as it is thought to depress ventilation. We found that in conscious rats [blood glucose] negatively correlated with baseline tidal volume. This negative correlation was also seen in V_T and V_E in response to hypercapnia but not in response to hypoxia, whereas hypoglycaemia had no effect on the ventilation of mice. Anaesthesia significantly depressed baseline ventilation and also blunted the response to hypercapnia and hypoxia in rats. There was a marked active expiration in hypercapnia that was not found in the hypoxic response.

4.1 The effect of hypoglycaemia on the ventilatory response to hypercapnia and hypoxia in rats

Plethysmography is a non-invasive indirect measurement of ventilatory variables giving readings that are unaffected by different variables such as anaesthesia and stress. It is clear that there is little if no stress to the animals during plethysmography as this study has shown there is no acclimatisation necessary to get a true baseline reading. When looking at the V_E of the sub group of rats that were given 5 days of acclimatisation it is possible to see there is no change between the 5 days. The low amount of cumulative stress and its non-invasive nature of this technique allowed animals to be their own internal control. Plethysmography also allowed us to conduct multiple exposures easily with software-controlled changes in gas content. The serial nature of these exposures had no effect on the resting V_E of the animals or on their response to the subsequent gas exposures.

Insulin-induced hypoglycaemia acts on peripheral and central glucoreceptors causing the sympathetic stimulation of the medulla and the release of adrenaline. Adrenaline acts on peripheral cells via ß-adrenergic receptors to increase metabolic rate, thereby increasing CO_2 production. To prevent an increase in P_aCO_2 an increase in ventilation must be linked to this hypermetabolic state. Insulin induced hypoglycaemia has been shown to cause an increase in resting minute ventilation in both anaesthetised rats (Bin-Jaliah, Maskell, and Kumar 2004, 2005) and humans (Ward, Voter, and Karan 2007). This was not found in the present study with there being no statistical difference between baseline and hypoglycaemic V_E. This combined with a lack of correlation between [blood glucose] and baseline V_E seems to disagree with the current literature. The lack of significance in V_E is down to a absence of change in frequency during hypoglycaemia. However there was a statistical difference in baseline tidal volume from fasted rats to hypoglycaemic rats and a negative correlation between [blood glucose] and V_T. Anatomical dead-space would be unchanged between experiments in the fasted and hypoglycaemic state and since alveolar ventilation equals $V_A = (V_T - V_{Dead\ space} \times f)$ the increased tidal volume with no change in R_f would lead to a raised alveolar ventilation thus increasing the efficiency of ventilation. Therefore there would be an increased gaseous exchange in hypoglycaemia; matching the raised CO_2 production in adrenaline induced hypermetabolism.

There was no was no correlation between the hypoxic V_E, V_T or R_f in rats with no increase in calculated O_2 sensitivity or correlation between [blood glucose] and

hypoxic minute ventilation. Data found by Ward et. Al. (2007) showed that using an intravenous hypoglycaemic clamp greatly increased the response to hypoxia in man, this is at odds with what we found in this study. This could show a species specific increase in O_2 sensitivity that is found in man but not in rats.

The R_f response to hypercapnia was also markedly decreased in hypoglycaemia due to an increased T_e. However, there was a correlation between blood glucose and hypercapnic V_T and V_E with a related significant increase in CO_2 sensitivity. It is thought that hypoglycaemia increases minute ventilation/alveolar ventilation through a carotid body mediated mechanism. Koyama et al. (2000) conducted hypoglyceamic clamp's on CBR and sham operated dogs and found that PCO_2 and HCO_3^- were both significantly higher in CBR whereas PO_2 was significantly lower. This showed a uncoupling of ventilation with metabolic rate which was then confirmed in their later studies (Koyama et al. 2001). Bin-Jaliah et al (2004) found CBR reduced basal V_E and prevented increased V_E in response to hypoglycaemia in rats.

The increase in CO_2 sensitivity but not in O_2 could point towards hypoglycaemia acting centrally through either a direct mechanism such as glucose sensitive cells of the nucleus solitary tract or through some related metabolic factor (Bin-Jaliah, Maskell, and Kumar 2004). Alternatively it could also show that there is a divergence in the sensing pathway for O_2, CO_2 and hypoglycemia in the carotid body. The carotid body is key to the hypercapnic response as carotid body resection causes a 30% reduction in normal hypercapnic response in humans (Lugliani et al. 1971), and an 80% reduction in the ventilatory response to central hypercapnia when silenced with

hyperoxia in animals (Blain et al. 2010). Bin-Jaliah et al. (2005) carotid body resection experiment showed that approximately 75% of the increase in CO_2 chemosensitivity during hypermetabolism arises from the carotid body. Recently Smith (2015) showed that stimulating a vascularly-isolated carotid body leads to an increased the central sensitivity to hypercapnia. Due to the neuronal make up of the central chemoreceptor region an excitatory input from the carotid body would prime central chemoreceptors causing a much more powerful hypercapnic response (Guyenet 2014), with carotid body stimulation increasing the response by roughly two fold (Blain et al. 2010).

Exposure to hyperoxic air causes a depression of tidal volume and respiratory frequency for 5-60 s (Zapata 2009). This hyperoxic response was not observed in normoglycaemic or fasted rats with both an increased V_E due to a increased frequency and unchanged tidal volume during exposure. This is due to methodological limitations with the short exposure time in order to observe the hyperoxic effect there was a lot of behavioral interference through the rats sensing the change in airflow in the chamber. During hypoglycemia the behavioral response was removed due to the subdued nature caused by low blood glucose making it was possible to see a decrease in V_E, R_f and V_T in response to hyperoxia. Evidence from the literature points towards two effects could have been seen if normoglycaemic and fasting results were positive. Firstly we could have seen a greater drop in ventilation in hypoglyceamic compared to normoglycaemic response, which would have agreed with Wehrwein et al. (2010) results that saw a blunting of hypoglycaemic response with hyperoxic silencing of the carotid body. Suggesting that hypoglycaemic

hyperventilation was carotid body mediated. On the other hand it is possible that there would not be statistically different drop in minute ventilation normoglycaemia compared to hypoglycaemia. This is possible as Ward et al. (2009) reported that hyperoxia only caused a slight reduction in the hypoglycaemic response that they put down to increased insulin sensitivity and not to carotid body activity. This would show that there was a carotid body independent role in hypoglycaemic hyperventilation.

In conscious rats there was an increase in CO_2 sensitivity but not in O_2 sensitivity, which leads to the conclusion that there are couple of alternative mechanisms for the hyperpnoea in hypoglycaemia. Firstly there must be a central mediated mechanism in which the sensing of low blood glucose alters the central CO_2 sensitivity. Secondly that via a carotid body mediated mechanism with a divergence in the carotid body sensing pathways for O_2, CO_2 and hypoglycemia.

4.3 The effect of hypoglycaemia on the ventilatory response in mice

During this study we have shown that hypoglycaemia causes an increase in baseline alveolar ventilation and CO_2 sensitivity in rats. However this was not the case in mouse experiments. There was a normal ventilatory response of increased V_E, V_T and R_f to hypercapnia and hypoxia in normoglycaemia and fasted mice however hypoglycaemic mice mounted no significant response to either hypoxia or hypercapnia.

This could be due to the extreme hypoglycaemic levels that were obtained in the mice experiment. When looking at the individual break down of blood glucose levels it was clear to see that mouse 2 and 5 had the highest blood sugar during testing with levels of 3.9 and 5.3 mmol/L respectively, whereas the others had blood sugars ranging from 3.2 to 1.8. This combined with the change in correlation between blood glucose & V_E when the extreme lows were removed from the regression analysis may suggest that there is a threshold below which blood sugar goes too low to trigger a significant ventilatory response to hypercapnia.

Alternatively this could be due to the difference in counter regulatory responses in C57BL/6 mice compared to rats. Rats mount a counter regulatory response to hypoglycaemia, which is consistent with that found in humans with the release of glucagon, noradrenaline, cortisol and adrenaline (Borg et al. 1994). However C57BL/6 mice do not mount an adrenaline response to hypoglycaemia (Berglund et al. 2008) .This lack of adrenaline response to hypoglycaemia is crucial as adrenaline is the most likely candidate for the metabolic factor involved in the hyperventilatory response to hypoglycaemia, as it is known to be involved released during both exercise and hypoglycaemia (Koyama et al. 2000, Koyama et al. 2001).

Adrenaline's role could be via a carotid body or central mediated mechanism. Heistad, Wheeler et al. (1972) found infusion of adrenaline increases minute ventilation in man. This increase is removed when the carotid body is silenced through application of 100% oxygen, thus points towards a carotid body mediated mechanism. However, Joels and White (1967) saw the opposite with intravenous

catecholamine infusions producing an increase in ventilation and carotid body chemoreceptor discharge after both aortic nerves and both cervical sympathetic nerves had been cut, suggest a carotid body independent role for adrenaline.

Hypoglycaemia in mice that has failed to cause a significant increase in baseline hypoxic or hypercapnic responses supports the idea that low glucose does not have a direct action on the carotid body. Some *in vivo* studies in the literature suggests that the carotid body is directly sensitive to hypoglycaemia (Koyama et al. 2000, Koyama et al. 2001, Wehrwein et al. 2010) which was also confirmed by *in vitro* studies (Alvarez-Buylla and de Alvarez-Buylla 1988) however this study disagrees with the studies such as Holmes, Turner et al. (2014) who found that glycogen stores in type 1 cells are protective against low [blood glucose] showing hypoglycaemia can not act directly on the carotid body.

The lack of a hyperpnoea in response to hypoglycaemia in mice that do not produce adrenaline response to hypoglycaemia hints at the role of adrenaline in matching ventilation to metabolism. It also can be concluded that hypoglycaemia does not act directly on the carotid body

4.4 Differences in hypoxic response in mice and rats

When looking at the responses to hypoxia in mice and rats it was clear to see that mice showed a much smaller response to the same level of hypoxia than rats, this difference in size of response points towards a difference in the hypoxic sensitivity in different species. It has been shown that small mammals respond to exposure to hypoxia through a reduction in their basal metabolic rate instead of a raised

ventilatory rate, this hypometabolic state is linked heavily to the size and age of the animal with a increase in weight and age in rats being inversely correlated with the decrease in metabolism (Mortola et al. 1994). This finding in consistent with the results presented here as the rats used in the present study were of similar weight to the heaviest used in Mortola et al. (1994) study. The correlation between the size of an animal and the level of hypometabolism was also found by Frappell et al.(1992) who reported a larger hypometabolic response in smaller animals, because of their higher thermogenic requirements. The decreased hypoxic response found in mice during the present study therefore may be due to a induction of a hypometabolic state.

4.5 Anaesthesia's effect on the response to hypercapnia and hypoxia in rats

The studies we have conducted on anesthetised rats have shown that hypoglycaemia causes a significant increase in baseline, hypoxic and hypercapnic tidal volume. This rise in tidal volume like in plethysmography studies without a concurrent rise in minute ventilation again could show that a rise in alveolar ventilation is sufficient to maintain a stable $PaCO_2$ in hypermetabolic state. However due to the lack of significant minute ventilatory response to both hypercapnia and hypoxia in normoglycaemia it isn't possible to say if there has been an increase in either CO_2 or O_2 sensitivity. This lack of normoglycaemic response can be attributed to the ventilatory depressive effect that anaesthesia can have. When comparing the baseline respiratory frequency from the plethysmography study to that found in anaesthetised experiments it is possible to see that there is a decrease in mean respiratory frequency. This reduction in respiratory frequency was also found in

Alfaxan anaesthetised cats who saw a greater than 50% decrease in respiratory frequency (O'Hagan et al. 2012). The effect of anaesthesia can also be found in the size of the response to hypercapnia and hypoxia, in plethysmography experiments respiratory frequency has increased by around 50% whereas there is only around a 10% increase during anaesthesia. Gautier (1976a) found this loss of sensitivity to hypoxia and hypercapnia in anaesthetised cats compared to conscious controls. In conclusion there is significant evidence to show that anaesthesia has a depressive effect on ventilation

However, there have been similar experiments that did see a hypoxic response in anaesthetised rats. Neylon (1991) showed a distinctive hypoxic response in Saffan (Alfaxan) anaesthetised rats with further experiments from the same group also agreeing with this (Thomas 1994). The hypoxic responses in these study's show the variation in response that animals under anaesthesia can develop with response depending on the anaesthetic used, depth of anaesthesia and species that is used. This variation confirms the need for methods such as plethysmography that monitor ventilation in conscious animals.

4.6 Different patterns of breathing during hypoxia and hypercapnia

It was hypothesised that there would be an active expiration during hypercapnia that would not be found in hypoxia. This hypothesis was presented as there was increases in abdominal EMG activity during hypercapnia, with the decrease in T_e accounting for 65-100% of the decrease in cycle length during hypercapnia (Gautier, Remmers, and Bartlet 1973). This combined with more recent work by Jenkin and Milsom (2015) who showed that there was active expiration at 8% and 10%

hypercapnia in conscious rats but not anaesthetized. This active expiration was not seen during any level of hypoxia. By measuring the change in T_i:T_e it is possible to show the amount of frequency increase that is due to decrease in expiratory time.

The example human active expiration shows that T_e is significantly decreased in an active expiration to such an extent that it is near to T_i values. By comparing T_i to T_e through a ratio it is possible to see how close the two values are. If T_e is close or the same as T_i then there must be an active process to move the same amount of air in the same time as the active process of inspiration. Although there was still a significant increase in T_i:T_e ratio during hypoxia this could still be through a passive process of increased alveolar recoil (Hlastala and Berger 2001). Despite rising to the sane level of tidal volume in both hypercapnia and hypoxia there is still a large decrease in T_e during hypercapnia which must be due to an active expiration.

The increased change in T_i:T_e in hypercapnia with hypoglycemia shows an greater active expiration during hypoglycemia. This could again point towards an increase in CO_2 sensitivity during hypoglycemia as central chemoreceptor region of the retrotrapezoid nucleus overlaps with the parafacial respiratory group which is solely responsible for the activation of expiratory muscles and could provide the CO_2-dependent excitatory drive that enables this oscillator to be active (Guyenet 2014).

4.7 Methodological limitations

There are some recognized limitations to the experiments carried out in this study:

Firstly there was no control insulin tolerance curve conducted so the exact level of blood glucose during exposures is unknown. However when looking at the available literature it seems that rats blood glucose plateaus 30mins post insulin injection and does not begin to rise until after 120mins Kinzig, Honors et al. (2010) in which time our protocol would be over. We also conducted a test on 2 anaesthetised rats in which we found a 1IUKg^{-1} dose of insulin was not enough to get a sufficient level of hypoglycemia hence why the dose was increased to 1.5IUKg^{-1}. The response timings (30-120mins) were still maintained even in this raised dose.

Due to the mechanism by which gasses are introduced into the plethysmography chamber the change in gas contents is not immediate. The rat plethysmography chamber had a total capacity 3.8L with the recommended gas flow of 2Lmin^{-1} it would take around 2mins for the chamber to equilibrate to the exact gas mixture. This should not have affected the data provided as taking the last minute of the 5min exposure still allows for a 3min exposure time to the required gas mixture.

It cannot be said that the raised ventilation was matched to metabolism, as we were unable to take blood gas readings from anesthetised rats or measure the expired CO_2 in conscious rats. However the literature has provided enough evidence that this is the case (Bin-Jaliah, Maskell, and Kumar 2004, 2005) should be sufficient for us to conclude that this is the case. In future studies it would be possible to measure show that ventilation matched metabolism in conscious rats by measuring the expired CO_2.

When inducing hypoglycaemia in mice [blood glucose] dropped down to levels that were lower than anticipated and continued to fall throughout the protocol. Blood glucose dropped to levels where insufficient glucose was available for normal cellular metabolism. This could be the case as when looking at the regression between [blood glucose] and minute ventilation the regression analysis on the full data had a unanticipated however the reduced data set had regression analysis similar to what we hypothesised. This may show that there is a threshold below which it is no longer possible to respond to hypercapnic/hypoxic stimulus. In two cases the experiments had to be stopped prematurely to administer glucose. In most cases however the lowest blood glucose achieved was 1.9mmolL^{-1}, at this level Berglund et al.(2008) still saw a normal counter regulatory response and once the test was stopped mice returned to normal with in 30-60mins. However in future the dose would be reduced to try and prevent the continued fall in blood glucose seen through the exposure protocol, as it is difficult to say what level blood glucose would have been at the time of exposure.

4.8 Future work

Based on the findings of the current study, several pieces of future work could be carried out to help consolidate the conclusions and address the ideas raised by the data:

Repeating hyperoxia experiments would be beneficial to this study as hyperoxia is a non-invasive way to silence the carotid body. To overcome the behavioural response that the animals showed to the influx of gas into the chamber it would be possible to set a gas flow of normal air into the chamber until they acclimatise to the gas flow,

after which the gas mixture could be changed to hyperoxic levels. Alternatively the carotid body input could be removed using CSNX or CBR, surgically removing CB input would allow comparisons with the current results leading to the identification of the role in the carotid body in hypoglycaemia induced hyperpnoea.

The use of an adrenaline receptor antagonist during hypoglycaemic readings would show the role that adrenaline has in hypoglycaemia hyperventilation and in combination with CBR studies would allow the identification of the site that adrenaline acts to increase CO_2 sensitivity.

The lack of a ventilatory response to hypoglycaemic in mice experiments points towards adrenaline as the cause of hyperpnoea during hypoglycaemia. Repeating these experiments with a mice strain that does produce adrenaline in response to hypoglycaemia will allow us to draw comparisons and confirm the role of adrenaline.

5. Conclusions

The increase in baseline alveolar ventilation and hypercapnic response in conscious rats during hypoglycaemia has shown that hypoglycaemia increases CO_2 sensitivity. When comparing this to C57BL/6 mice that did not show a ventilatory response to hypoglycaemia, we have attributed this to the fact that they do not mount an adrenaline response to hypoglycaemia. This suggests firstly that there is no direct sensing of low [blood glucose] by the carotid body and secondly that adrenaline has a role in the hyperpnoea in hypoglycaemia. Whether increase CO_2 sensitivity is due to adrenaline acting on the carotid body or if it is sensitisation of central chemoreceptors by hypoglycaemia.

We have shown the depressive effects that anaesthesia can have on the ventilatory response with whole body plethysmography a reliable replacement to identify the effect that hypoglycaemia has on the ventilatory response to hypercapnia and hypoxia. With the help of some of the future advancements mentioned, this study could be the basis of identifying the mechanism for the hyperpnoea during hypoglycaemia.

Finally the marked increase in T_i:T_e in hypercapnia points towards an active expiration during hypercapnia which is not present during hypoxia building on the current evidence that the pattern of breathing is different in the response to hypoxia and hypercapnia with a pronounced active expiration in hypercapnia. This raises

questions as to how different stimuli are processed to produce a different pattern change in ventilation.

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